PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/68

A2

(11) International Publication Number: WO 98/14611

(43) International Publication Date: 9 April 1998 (09.04.98)

(21) International Application Number: PCT/US97/17734

(22) International Filing Date: 1 October 1997 (01.10.97)

(30) Priority Data:
60/027,353 2 October 1996 (02.10.96) US

(71) Applicant *(for all designated States except US)*: THE GOVERNMENT OF THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; The Centers for Disease Control and Prevention, 1600 Clifton Road N.E., Atlanta, GA 30333 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: KILPATRICK, David [US/US]; 1095 Fulton Court, Norcross, GA 30093-4041 (US).

(74) Agents: SAMPLES, Kenneth, H. et al.; Fitch, Even, Tabin & Flannery, Suite 900, 135 South LaSalle Street, Chicago, IL 60603-4277 (US).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

**Published**
*Without international search report and to be republished upon receipt of that report.*

(54) Title: DETECTION AND IDENTIFICATION OF NON-POLIO ENTEROVIRUSES

(57) Abstract

This invention provides sensitive nucleic acid hybridization assay methods and kits for the detection of non-polio enterovirus nucleic acids. The methods are particularly useful in detecting the presence of enterovirus nucleic acids in a biological sample, and for ascertaining the serotype of enteroviruses present in a sample.

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| AL | Albania | ES | Spain | LS | Lesotho | SI | Slovenia |
| AM | Armenia | FI | Finland | LT | Lithuania | SK | Slovakia |
| AT | Austria | FR | France | LU | Luxembourg | SN | Senegal |
| AU | Australia | GA | Gabon | LV | Latvia | SZ | Swaziland |
| AZ | Azerbaijan | GB | United Kingdom | MC | Monaco | TD | Chad |
| BA | Bosnia and Herzegovina | GE | Georgia | MD | Republic of Moldova | TG | Togo |
| BB | Barbados | GH | Ghana | MG | Madagascar | TJ | Tajikistan |
| BE | Belgium | GN | Guinea | MK | The former Yugoslav Republic of Macedonia | TM | Turkmenistan |
| BF | Burkina Faso | GR | Greece | | | TR | Turkey |
| BG | Bulgaria | HU | Hungary | ML | Mali | TT | Trinidad and Tobago |
| BJ | Benin | IE | Ireland | MN | Mongolia | UA | Ukraine |
| BR | Brazil | IL | Israel | MR | Mauritania | UG | Uganda |
| BY | Belarus | IS | Iceland | MW | Malawi | US | United States of America |
| CA | Canada | IT | Italy | MX | Mexico | UZ | Uzbekistan |
| CF | Central African Republic | JP | Japan | NE | Niger | VN | Viet Nam |
| CG | Congo | KE | Kenya | NL | Netherlands | YU | Yugoslavia |
| CH | Switzerland | KG | Kyrgyzstan | NO | Norway | ZW | Zimbabwe |
| CI | Côte d'Ivoire | KP | Democratic People's Republic of Korea | NZ | New Zealand | | |
| CM | Cameroon | | | PL | Poland | | |
| CN | China | KR | Republic of Korea | PT | Portugal | | |
| CU | Cuba | KZ | Kazakstan | RO | Romania | | |
| CZ | Czech Republic | LC | Saint Lucia | RU | Russian Federation | | |
| DE | Germany | LI | Liechtenstein | SD | Sudan | | |
| DK | Denmark | LK | Sri Lanka | SE | Sweden | | |
| EE | Estonia | LR | Liberia | SG | Singapore | | |

# DETECTION AND IDENTIFICATION OF NON-POLIO ENTEROVIRUSES

## BACKGROUND OF THE INVENTION

### A. Field of the Invention

The invention relates to the detection and classification of pathogenic viruses. In particular, the invention provides diagnostic assays for the detection and classification of enterovirus nucleic acids in biological and other samples.

### B. Related Art

Enteroviruses are a heterogeneous group of pathogens responsible for a broad spectrum of human and nonhuman diseases. Enteroviruses belong to a large genus within the family *Picornaviridae*; other genera within this family include rhinoviruses, hepatoviruses, cardioviruses, and aphthoviruses. The enterovirus genus encompasses polio viruses, coxsackie A viruses (CAV), coxsackie B viruses (CBV), echoviruses, and enteroviruses 68-71, as well as a number of uncharacterized enteroviruses isolated from humans and other primates. (For a review of taxonomy of *Picornaviridae see*, VIRUS TAXONOMY: CLASSIFICATION AND NOMENCLATURE OF VIRUSES Murphy *et al.*, eds. Springer Verlag, 1995).

#### 1. Biological properties of enteroviruses

Like other picornaviruses, enteroviral virions comprise an icosahedral capsid, about 30 nm in diameter, with no envelope, enclosing a core comprising infectious, singl - strand d genomic sense RNA, about 7-8.5 kb in size. Enteroviruses are distinguish d from other members of the picornaviridae by their stability in acid and their fecal-oral route of passage and transmission. Virus entry into cells is believed to involve specific cellular receptors.

Virion proteins include multiple copies of four capsid proteins (P1 gene products IA, IB, IC, ID such as poliovirus VP4, VP2, VP3, VP1, respectively. A small protein, VPg (Mr about 24 x $10^3$), is linked covalently to the 5' terminus of the genomic RNA.

The viral genome consists of a ssRNA with a 5' untranslated sequence of variable length followed by an ORF encoding the polyprotein precursor (Mr 240-250 x $10^3$) to the structural proteins (P1) and the predominantly nonstructural proteins (P2, P3), followed by a short non-coding sequence and a poly (A) tract of variable length. Fig. 1 depicts a generalized enteroviral genome. The filled circle at the 5' end is the genome-linked protein VPg (also referred to as the 3B gene product), followed by, the 5' non-translated region (line). The open boxes depict a long open reading frame encoding a polyprotein that is split to yield the individual proteins mentioned above, followed by the 3' non-translated region (line) and a poly (A) track (angled line). The eventual cleavage products of the polyprotein are indicated by vertical lines in the boxes, the nomenclature of the polypeptides follows an L:4:3:4 scheme corresponding to the genes (numbers) encoded in the L, P1, P2, P3 regions (Rueckert and Wimmer, 1984). The P1 region encodes the structural proteins 1A, 1B, 1C and 1D, usually referred to as VP4, VP2, VP3, and VP1, respectively. VP0, not shown here, is an intermediate precursor for VP4 and VP2. In all viruses, 3C is a protease, in enteroviruses and rhinoviruses 2A is a protease, while in all viruses 3D is considered to be a component of the RNA replicase.

The serotype designations (in parenthesis) of a number of enteroviruses and their genomic sequence accession numbers [in brackets] are:

| | | |
|---|---|---|
| bovine enterovirus 1 | (BEV-1) | [D00214] |
| bovine enterovirus 2 | (BEV-2) | |
| human coxsackievirus A1 to 22 | (CAV-1 to 22) | [D00538] |
| human coxsackievirus A24 | (CAV-24) | |
| human coxsackievirus B I to 6 | (CBV-1 to 6) | [M33854] |
| human echovirus 1 to 7 | (EV-1 to 7) | |
| human echovirus 9 | (EV-9) | |
| human echovirus 11 to 27 | (EV-11 to 27) | |
| human echovirus 29 to 33 | (EV-29 to 33) | |

| | | |
|---|---|---|
| human enterovirus 68 to 71 | (HEV68 to 71) | |
| human poliovirus 1 | (HPV-1) | [V01150] |
| human poliovirus 2 | (HPV-2) | |
| human poliovirus 3 | (HPV-3) | |
| porcine enterovirus 1 to 11 | (PEV-1 to 11) | |
| simian enterovirus 1 to 18 | (SEV-1 to 18) | |
| Vilyuisk virus | | |

Sequence identities for different enteroviruses, or between enteroviruses and rhinoviruses are more than 50% over the genome as a whole. Strains within a species often have more than 75% sequence identity over the genome as a whole. Viruses grouped by biological criteria, *e.g.*, the polioviruses, or Coxsackie B viruses, are generally closely related in terms of overall nucleotide sequence identity over the genome as a whole. Different enteroviral serotypes are classified by cross-protection neutralization of infectivity, complement-fixation, specific ELISA using a capture format, or immunodiffusion. Some species can be identified by hemagglutination.

The following is a partial listing of reported correlations between enteroviral species and diseases (Morens, *et al.*, *Textbook of Human Virology*, pp. 427-497, 2nd ed., Mosby-Year Book, St. Louis (1991); Grandien, *et al.*, *Diagnostic Procedures for Viral, Rickettsial and Chlamydial Infections*, pp. 513-569, 6th ed. American Public Health Association, Washington, D.C. (1989)):

<u>Poliovirus</u>

| | | | | | |
|---|---|---|---|---|---|
| PV1 | PM | AFP | AM | C* | URI* |
| PV2 | PM | AFP | AM | C* | URI* |
| PV3 | PM | AFP | AM | C* | URI* |

<u>Coxsackievirus A</u>

| | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| CA01 | AM* | ABP* | Ena* | | | | | | | |
| CA02 | AFP* | AM* | Enc* | Ena | Ex* | | | | | |
| CA03 | AM* | Ena* | | | | | | | | |
| CA04 | AFP* | AM* | Enc* | NND* | C* | Ena | HFM* | Ex* | Pl* | |
| CA05 | AM* | Enc* | Ena | HFM* | Ex* | | | | | |
| CA06 | AM* | Enc* | Ena | Pl* | | | | | | |
| CA07 | AFP* | AM* | Ena* | LRI | | | | | | |
| CA08 | AFP* | AM* | Ena | | | | | | | |
| CA09 | AFP* | AM | Enc | NND* | ABP* | Ena* | Ex* | Pl* | LRI* | Cr |
| CA09var | HFM | | | | | | | | | |
| CA10 | AFP* | AM* | Ena | HFM* | Pl* | URI | | | | |

| | |
|---|---|
| CA11 | AM* |
| CA12 | |
| CA13 | |
| CA14 | AM* C* |
| CA15 | |
| CA16 | AFP* AM* NND* C* Ena HFM Ex* |
| CA17 | AM* |
| CA18 | AM* |
| CA19 | Guillain-Barré |
| CA20 | Hep |
| CA21 | URI |
| CA22 | AM* Ena* GI |
| CA24 | AM* URI |
| CA24var | AHC |
| CB1 | AM AFP* Enc* NND M ABP Ena* Ex* Pl URI* LRI* |
| CB2 | AM AFP Enc* NND M ABP Ena* Ex* Pl URI |
| CB3 | AM Pl AFP Enc* NND M ABP Ena* Ex* Pl URI* |
| CB4 | AM AFP Enc* NND M ABP Ena* Ex* Pl URI* LRI* |
| CB5 | AM M AFP Enc NND M ABP Ena* HFM* Ex* Pl Cr |
| CB6 | AFP* AM* Pl |

<u>Echovirus</u>

| | |
|---|---|
| E01 | AFP* AM* NND* ABP* Ex* Pl* URI |
| E02 | AFP* AM* Enc* NND* Ex* |
| E03 | AM AFP Enc* NND* Ena* Ex* URI* |
| E04 | AM AFP Enc* NND* C* Ex* URI* |
| E05 | AM* NND* Ex* |
| E06 | AM AFP Enc* NND* C* Ena* Ex* Pl* URI* |
| E06' | |
| E06" | AM |
| E07 | AM AFP* Enc* NND* C* URI* |
| E08 | ABP* |
| E09 | AM AFP Enc NND C* ABP* Ena* Ex LRI* |
| E11 | AM AFP Enc* NND Ex* Pl* URI* Cr |
| E11' | AM |
| E12 | |
| E13 | AFP* |
| E14 | AM* Enc* NND Ex* |
| E15 | NND* |
| E16 | AFP* AM* NND* Ena* BE Ex* |
| E17 | NND* Ena* |
| E18 | AFP* Enc NND Ex* GI |
| E19 | AM* AFP* Enc* NND C* ABP* Ex* Pl* URI* GI |
| E20 | AFP* C URI* |
| E21 | NND* |
| E22 | AM* NND URI* GI |
| E23 | GI |
| E24 | AM* |
| E25 | Enc* Ex* URI* |
| E26 | |
| E27 | AM* |

| | | | |
|---|---|---|---|
| E29 | | | |
| E30 | AFP* | AM* | Ena* |
| E31 | AM | NND* | |
| E32 | AM | | |
| E33 | AM* | | |
| E34 | GI | | |

<u>Other enteroviruses</u>

| | | | |
|---|---|---|---|
| EV68 | LRI* | | |
| EV69 | | | |
| EV70 | AHC | | |
| EV71 | Pa | AM | HFM* |

ABP-acute benign pericarditis, AFP-acute flaccid paralysis, AHC-acute hemorrhagic conjunctivitis, AM-aseptic meningitis, BE-Boston exanthema, C-carditis, Cr-croup, Enc-encephalitis, Ena-enanthema, Ex-exanthema other than BE or HFM, GI-gastrointestinal disease, Hep-hepatitus, HFM-hand-foot-and-mouth disease, LRI-lower respiratory infection, M-myocarditis, NND-neonatal disease, Pe-pericarditis, Pl-pleurodynia, PM-poliomyelitis, Ra-rash, RD-respiratory disease, UF-undifferentiated fever; URI-upper respiratory infection; *infrequent association.

Other possible associations: nonspecific febrile illness; fatigue syndrome; gastrointestinal disease; hepatitis; diabetes mellitus; pancreatitis; urinary tract infection; arthritis; hemolytic uremic syndrome; orchitis; et al.

Polioviruses (which exist as at least three serotypes) are the most clinically significant of the enteroviruses worldwide, causing paralytic disease in children in developing countries.

Non-polioenteroviruses (NPEV) are also responsible for large numbers of symptomatic and asymptomatic infections each year. Data suggests that there are between 10-15 million illnesses due to NPEV infections each year in the United States (Strikes *et al.*, 1986). NPEVs are responsible for 30,000-50,000 hospitalizations each year for aseptic meningitis, myocarditis, encephalitis, acute hemorrhagic conjunctivitis, nonspecific febrile illnesses, and upper respiratory tract infections (Melnick, *Biologicals 21*:305-309 (1993)). Certain forms of insulin-dependent diabetes mellitus, affecting an estimated 1 million individuals in the U.S. alon , with 100,000 newly diagnosed each year, may be caused by Cocksackies B4 and B5 virus and echovirus 18. (Wagenknecht *et al.*, *Amer. J. Epidem. 133(10)*:1024-1031 (1991); Frisk *et al.*, *J. of Infection 24(1)*:13-22 (1992).)

Enteroviruses are also associated with acute flaccid paralysis: CAVs caused flaccid paralysis in newborn mice, whereas CBV infection in mice resulted in spastic paralysis. Enteroviruses are also associated with dilated cardiomyopathy (Cochrane *et al.*, 1991) and foot and mouth disease. Recent reports have linked NPEV infection with chronic fatigue syndrome (Clements *et al. J. Med. Virol.* 45:156-161 (1995).

**2. Detection of nonpolio enteroviruses**

A full catalogue of nonpolio enteroviral serotypes and diseases, and the development of effective treatments for nonpolio enteroviral diseases, are severely limited by the lack of efficient, sensitive diagnostic assays for detecting and classifying enteroviruses. Problem diseases are not detected because there is no quick efficient method for the detection and identification of nonpolio enteroviral infection. Current clinical diagnosis usually relies on medical history and clinical examination. Laboratory methods are based on tissue culture isolation followed by micro-neutralization tests using specific antisera. Each enterovirus generally requires a different array of cell lines. The assays may take weeks and have less sensitivity than biological reagents.

Probe hybridization assays (using either cDNA or RNA probes) have also been used to detect NPEVs (Rotbart *et al.*, *Mol. Cell. Probes 2*:65-73 (1988); Rotbart, *J. Clin. Microbiol. 28*:438-442 (1990); Chapman *et al.*, *J. Clin. Microbiol. 28*:843-850 (1990); differing Hyypiä, *et al.*, *J. Gen. Virol. 70*:3261-3268 (1989); Olive *et al.*, *J. Gen. Virol. 71*:2141-2147 (1990); Gilmaker *et al.*, *J. Med. Virol. 38*:54-61 (1992); Yang *et al.*, *Virus Res. 24*:277-296 (1992); Zoll *et al.*, *J. Clin. Microbiol. 30*:160-165 (1992); Muir *et al.*, *J. Clin. Micro. 31*:31-38 (1993); Drebot *et al.*, *J. Med. Virol. 44*:340-347 (1994); Rotbart et al., *J. Clin. Microbiol. 32*:2590-2592 (1994)). Due to the lack of enteroviral nucl ic acid sequence information, most of these probes have targeted the highly conserved 5' non-coding region of the viral genomes. Rotbart, *et al.*, *Human Enterovirus Infections*, pp. 401-418 (1995). First, this

region is reportedly very important for the proper translation of the viral genome and s cond, it is a noncoding region and thus not subject to the degeneracy found in a coding region. Furthermore, RNA probes which target the VP1 capsid region have been used on a limited basis to identify some of the CBVs and a few closely related CAVs (Cova *et al., J. Med. Virol.* *24*:11-18 (1988); Alksnis *et al., Mol. Cell. Probes* *3*:103:108 (1989); Petitjean *et al., J. Clin. Microbiol.* *28*:307-311 (1990)).

None of the published methods for detecting enteroviruses (see a review by Rotbart, *et al., Human Enterovirus Infections*, pp. 401-418 (1995)) can differentiate among the subgroups and serotypes of enteroviruses (*i.e.*, CAV types 1-22, 24; CBV types 1-6, echovirus types 1-9, 11-21, 24-27, 29-33; enteroviruses 68-71). A detection system that identifies and differentiates most or all enterovirus serotypes would improve the speed and accuracy of processing samples and increase the sensitivity of detecting minority populations of enteroviruses in mixed serotype cultures. For example, a prime target in enterovirus diagnosis is to determine their presence, or absence, in newborn infections in order to ascertain whether a meningitis infection is bacterial or viral in nature (Abzug *et al., J. of Pediatrics* *126*:447-450 (1995); Rotbart, *et al., Human Enterovirus Infections*, pp. 401-418 (1995)). In addition, rapid and efficient detection and identification of contaminating enteroviruses in vaccine preparations such as polio vaccine preparations is important to ensure the safety of viral vaccines. The present invention addresses these and other concerns.

## SUMMARY OF THE INVENTION

The present invention provides methods for detecting and serotyping nonpolio enterovirus (NPEV) nucleic acids in biological samples and in vaccine preparations such as polio vaccines. In one embodiment, the invention provides prim rs useful for detecting a nonpoliovirus enterovirus. In another embodiment, the invention provides methods for detecting

recombinant viral nucleic acids which include nucleic acid s quenc s from a polio virus and a non-polio enterovirus.

In some embodiments, the invention comprises pairs of primers used to detect the presence or absence of a nonpolio enterovirus in a sample and to identify nonpolio enterovirus serotypes, wherein a first primer of a pair binds to a sense strand of a first nonpolio enterovirus nucleic acid sequence that encodes a first conserved nonpolio enteroviral peptide sequence, and a second primer of the pair binds to an antisense strand of a second nonpolio enterovirus nucleic acid sequence that encodes a second conserved nonpolio enteroviral peptide sequence, wherein both conserved sequences are from a same nonpolio enteroviral protein, to yield an amplification product of a nonpolio enteroviral sequence that encodes both the first and second conserved nonpolio enteroviral peptide sequence.

The methods of the invention also comprise assays for detecting the presence or absence of a nonpolio enterovirus nucleic acid sequence in a sample, comprising contacting the sample with a first and second pair of oligonucleotide primers in an amplification protocol, and determining the presence or absence of a nonpolio enterovirus by detecting for the presence or absence of amplification products.

The methods further comprise detecting recombination between different enteroviruses by contacting a sample suspected of containing an NPEV nucleic acid with a first primer which specifically hybridizes to a conserved sequence in an enteroviral genome and a second primer which specifically hybridizes to a second enteroviral nucleic acid sequence. The presence of an amplified product which is a recombinant viral nucleic acid is then detected.

The methods are performed using samples commonly used for clinical analysis of nucleic acids. A typical sample is a biological sample, such as human serum.

The invention also provides methods for detecting a nonpoliovirus nucleic acid in a vaccine preparation such as a polio vaccine. Th methods comprise contacting the vaccine

sample with at least two primers which specifically hybridize to NPEV nucleic acid sequences. NPEVs may optionally be detected using gel electrophoresis to identify an amplified fragment that is not present in a control vaccine sample known to contain only poliovirus nucleic acids.

Using 15 complete VP1 sequences, a series of NPEV amplification assay primers were designed to match intervals encoding amino acid sequences within VP1 that are strongly conserved among NPEVS. These primers contain mixed-base and deoxyinosine residues to compensate for the high degeneracy of the targeted codons. Primer sets were identified that code for amino acid sequences which are uniquely conserved among individual groups and serotypes of enteroviruses.

A series of 8 primer sets were preferred for selectively screening for the presence of NPEVs. These degenerate primer sets increase the speed and sensitivity of detecting NPEVs in clinical isolates. Even though no VP1 sequence information was available for the majority (35 out of 49 of enteroviruses tested, surprisingly, 48 out of 49 different enterovirus serotypes could be detected using the methods of the invention.

## BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the genome structure and gene organization of enteroviruses. The filled circle at the 5' end is the genome-linked protein VPg (also referred to as the 3B gene product), followed by the 5' non-translated region (5' NTR; solid line). The open box depicts the long ORF encoding the polyprotein that is followed by the 3' non-translated region (line) and a poly (A) track (angled line). The eventual cleavage products of the polyprotein are indicated by vertical lines in the boxes. The P1 region encodes the structural proteins VP4, VP2, VP3, and VP1, (also referred to as 1A, 1B, 1C and 1D, respectively.)

Figure 2 shows the alignment of corresponding amino acid residues within the VP1 proteins of 15 human enterovirus r ference strains. Abbreviations for virus groups are

followed by s rotype number: CAV, coxsackievirus A; CBV, coxsackievirus B; Echo, echovirus; EV, enterovirus.

Figure 3 shows the specific amplification of selected NPEVs representing each major NPEV group with the primers listed in Table 1. Clarified lysates of infected cell cultures (1 μl/reaction) were the source of templates for a polymerase chain reaction ("PCR") protocol. After 30 amplification cycles, DNA products were separated by electrophoresis on polyacrylamide gels and visualized by ethidium bromide staining. The presence of an amplification product with the predicted size is indicated by a plus sign (+) in the appropriate column for each isolate.

Figure 4 shows the selected primer sets that were tested against a collection of 49 NPEVS. The samples were analyzed as described in Fig. 3. The presence of a PCR product, corresponding to the correct size for each different primer set, is indicated with a plus sign. PCR reactions yielding either no product, or a product of incorrect size are indicated with minus signs.

The results from Fig. 4 are summarized in Figure 5. After analysis with the selected primer sets, samples can be quickly screened by comparing to this chart. This information gives you the most likely serotype or group of serotypes which may be present in the sample. Further screening, using conventional micro-neutralization tests, can then be performed on only those suspected serotypes. This will significantly reduce the number of micro-neutralization tests that need to be done, thus speeding up identification by eliminating unnecessary testing and conserving the limited amounts of Melnick antisera pools that are available.

## DETAILED DESCRIPTION OF THE INVENTION

### A. Definitions

The terms "hybridize(s) specifically" or "specifically hybridize(s)" refer to compl mentary hybridization between an oligonucleotide ( .g., a primer or labeled probe) and a target sequence. The term sp cifically embraces minor mismatches that can b accommodat d by reducing

the stringency of the hybridization media to achiev the desired priming for the PCR polymerases or detection of hybridization signal.

The term "biological sample" refers to a sample comprising any biological material (*e.g.*, biological fluids) containing nucleic acids. Biological samples will typically comprise whole blood, serum, urine, saliva, cerebrospinal fluid, semen, and the like.

"Nucleic acid" refers to a deoxyribonucleotide or ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, would encompass known analogs of natural nucleotides that can function in a similar manner as naturally occurring nucleotides.

The term "oligonucleotide" refers to a molecule comprised of two or more deoxyribonucleotides or ribonucleotides, such as primers, probes, nucleic acid fragments to be detected, and nucleic acid controls. The exact size of an oligonucleotide depends on many factors and the ultimate function or use of the oligonucleotide.

The term "primer" refers to an oligonucleotide, whether natural or synthetic, capable of acting as a point of initiation of DNA synthesis under conditions in which synthesis of a primer extension product complementary to a nucleic acid strand is induced, *i.e.*, in the presence of four different nucleoside triphosphates and an agent for polymerization (*i.e.*, DNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. A primer is preferably a single-stranded oligodeoxyribonucleotide. The appropriate length of a primer depends on the intended use of the primer but typically ranges from about 10 to about 30 nucleotides. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template but must be sufficiently complementary to specifically hybridize with a template. The t rm primer, when directed to a sequence that encodes a defined peptide sequence, specifically encompasses degenerate primers designed to identify conserved amino acid residues, in

which the third position of either (one or more) selected or all codons is substituted with mixed-base and/or deoxyinosine residues (Batzer *et al.*, *Nucleic Acid Res. 19*:5081 (1991); Ohtsuka *et al.*, *J. Biol. Chem. 260*:2605-2608 (1985); and Cassol *et al.*, 1992; Rossolini *et al.*, *Mol. Cell. Probes 8*:91-98 (1994)). When primer pairs are referred to herein, the pair is meant to include one primer which is capable of hybridizing to the sense strand of a double-stranded target nucleic acid (the "sense primer") and one primer which is capable of hybridizing to the antisense strand of a double-stranded target nucleic acid (the "antisense primer").

"Probe" refers to an oligonucleotide which binds through complementary base pairing to a subsequence of a target nucleic acid. A primer may be a probe. It will be understood by one of skill in the art that probes will typically substantially bind target sequences lacking complete complementarity with the probe sequence depending upon the stringency of the hybridization conditions. The probes are typically directly labelled (*e.g.*, with isotopes or fluorescent moieties) or indirectly labelled such as with biotin to which a streptavidin complex may later bind. By assaying for the presence or absence of the probe, one can detect the presence or absence of the target.

A "sequence specific to" a particular virus species or strain (*e.g.*, poliovirus) is a sequence unique to the species or strain, (that is, not shared by other previously characterized species or strains). A probe or primer containing a sequence complementary to a sequence specific to a virus will typically not hybridize to the corresponding portion of the genome of other viruses under stringent conditions (*e.g.*, washing the solid support in 2xSSC, 0.1% SDS at 70°C).

The phrase "conserved nonpolio enteroviral peptide sequenc " means that a p ptide sequence is specific for at least two nonpoliovirus enteroviral sequences, and is present on the corresponding protein of at least two different nonpolio enteroviruses. "A same nonpolio enteroviral protein" means that the conserved nonpolio enteroviral peptide

sequenc s to which the primer pairs specifically hybridize are pr sent on a same protein (*e.g.*, VPg, the polyprotein precursor, VP4, VP2, VP3, VP1, the 2A non-structural protein) of at least one nonpolio enterovirus.

"Amplification protocol" means an assay for amplifying a nucleic acid sequence, such as a PCR assay, a ligase chain reaction assay (LCR), $Q\beta$-replicase amplification, transcription amplification, and self-sustained sequence replication.

The phrase "bracket a nucleic acid sequence" means that primers that bind to opposite strands of a DNA molecule are so disposed that a polymerase chain reaction replicates the nucleic acid sequence between the two primer binding sites.

The term "substantially identical" indicates that two or more nucleotide sequences share a majority of their sequence. Generally, this will be at least about 66% of their sequence and preferably about 95% of their sequence. Another indication that sequences are substantially identical is if they hybridize to the same nucleotide sequence, preferably under stringent conditions (see, e.g., Sambrook *et al.*, *Molecular Cloning - A Laboratory Manual*, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York, 1985). Stringent conditions are sequence-dependent and will be different in different circumstances. Generally, stringent conditions are selected to be about 5°C lower than the thermal melting point (Tm) for the specific sequence at a defined ionic strength and pH. The $T_m$ is the temperature (under defined ionic strength and pH) at which 50% of the target sequence hybridizes to a perfectly matched probe. Typically, stringent conditions will be those in which the salt concentration is about 0.2 molar at pH 7 and the temperature is at least about 60°C.

**B. The invention**

The present invention is directed to the detection of nucl ic acids derived from non-polioenteroviruses (NPEV). In particular, the invention provid s sensitive methods (*e.g.*,

the polymerase chain reaction, PCR) for detecting NPEV and recombinant viruses potentially deriv d from polio vaccines.

Nucleotide sequences of various enteroviruses are available in the scientific literature and in databases such as GenBank (National Center for Biotechnology Information, Natl. Library of Medicine, National Institutes of Health, 8600 Rockville Pike, Bethesda, Maryland 20894). For instance, sequences of poliovirus types 1, 2 and 3 are disclosed in Toyoda *et al.*, *J. Mol Biol* 174: 561-585, (1984). Sequences of NPEVs (*e.g.*, VP1 nucleic acid and peptide sequences) are reported at the following Accession Nos. CAV2-L28146, CAV9-D00627, CAV16-U05876, CAV21-D00538, CAV24-D90457, CBV1-M16560, CBV3-M33854, CBV4-X05690, CBV5-X67706, EV70-D00820, EV71-U22521, Echo 6-U05851, Echo 9-X84981, Echo 11-X80059, Echo 12-X77708 Brown and Fallansch (1995), *Virus Res.* 39:195-205

Molecular reagents targeting capsid sequences should give identifications that correlate better with the most important biological properties of the virus (e.g., receptor specificity, serotype) than would reagents targeting other regions. However, the lack of a sequence database for most of the NPEVs in the VP1 region has led to a lack of methods targeting this area of the genome. Another factor contributing to this lack of methods is that there can be a wide nucleotide sequence variation (even between members of the same serotype). This high degree of nucleotide sequence diversity among enteroviruses is exacerbated by the evidence that polioviruses undergo frequent recombination (Cammack *et al.*, *Virology 167*:507-514 (1989); Furione *et al.*, *Virology 196*:199-208 (1993); Lipskaya *et al.*, *J. Med. Virol. 35*:290-296 (1991); Rico-Hesse *et al.*, *Virology 160*:311-322 (1987); Zheng *et al.*, *J. Infect. Dis. 168*:1361-1367 (1993)); genetic exchange may place poliovirus capsid sequences into genetic backgrounds derived from other polioviruses (Kew *et al.*, *N w Aspects of Positive-Strain RNA Viruses*, pp. 357-365, American Society of Microbiology, Washington, D.C. (1990); King *et al.*, *Nucleic Acids Res. 16*:11705-11723 (1988); Zheng *et al.*, *J. Infect. Dis. 168*:1361-1367 (1993)) or possible NPEVs (Furione

*et al.*, *Virology 196*:199-208 (1993)). Similar recombination and genetic exchange is susp cted for many of the NPEVS, but direct evidence is lacking.

To address these problems, very specific PCR primer pairs that, as a group, can identify almost all NPEV serotypes are provided below. This is achieved by targeting highly conserved amino acid regions and by using degenerate primers.

**1. PCR amplification assays**

A number of amplifiction protocols may be used, but a polymerase chain reaction ("PCR") is preferred. As noted above, the primers of the invention are typically used in PCR amplification of the target nucleic acid. The PCR process is well known in the art and is thus only briefly described herein. For a review of PCR methods and protocols, *see, e.g.*, U.S. Patent Nos. 4,683,195; 4,683,202; 4,965,188; and Innis, *et al.*, eds. *PCR Protocols. A Guide to Methods and Application* (Academic Press, Inc., San Diego, CA. 1990), each of which is incorporated herein by reference. PCR reagents and protocols are also available from commercial vendors, such as Roche Molecular Systems.

Because enteroviruses are RNA viruses, the first step in the amplification is the synthesis of a DNA copy (cDNA) of the region to be amplified. Reverse transcription can be carried out as a separate step, or in a homogeneous reverse transcription-polymerase chain reaction (RT-PCR), a modification of the polymerase chain reaction for amplifying RNA. Methods suitable for PCR amplification of enterovirus nucleic acids are described in Romero and Rotbart in *Diagnostic Molecular Biology: Principles and Applications* pp.401-406, Persing *et al.* eds., (Mayo Foundation, Rochester, MN 1993); Rotbart *et al.* U.S. Patent No. 5,075,212 and Egger *et al.*, *J. Clin. Microbiol.* 33:1442-1447 (1995)).

The primers used in the methods of the invention are preferably at least about 15 nucleotides to about 50 nucleotides in length, more preferably from about 15 nucleotides to about 30 nucleotides in length. If a probe is used to detect the amplification product, the primers are

selected from parts of the viral genomes that are upstr am and downstream from the probe.

Preferably, the primers target the sense or antisense strands of nucleotide sequences that encode particular conserved regions. Particular combinations of groups of primer pairs yield a matrix of amplification products that is used to detect and serotype nonpolio enteroviruses present in a sample. A preferred combination is one comprising the following primer pairs (described in greater below):

5S/6A (SEQ ID NO:49/SEQ ID NO:50),
7S/9A (SEQ ID NO:53/SEQ ID NO:55),
14S/11A (SEQ ID NO:57/SEQ ID NO:56),
51S/52A (SEQ ID NO:73/SEQ ID NO:74),
61S/68A (SEQ ID NO:78/SEQ ID NO:84),
64S/65A (SEQ ID NO:81/SEQ ID NO:82),
67S/1A (SEQ ID NO:83/SEQ ID NO:48), and
67S/8A (SEQ ID NO:83/SEQ ID NO:54).

To amplify a target nucleic acid sequence in a sample by PCR, the sequence must be accessible to the components of the amplification system. In general, this accessibility is ensured by isolating the nucleic acids from the sample. A variety of techniques for extracting nucleic acids, in particular ribonucleic acids, from biological samples are known in the art. Alternatively, if the sample is fairly readily disruptable, the nucleic acid need not be purified prior to amplification by the PCR technique, *i.e.*, if the sample is comprised of cells, particularly peripheral blood lymphocytes or monocytes, lysis and dispersion of the intracellular components may be accomplished merely by suspending the cells in hypotonic buffer.

The first step of each cycle of the PCR involves the separation of the nucleic acid duplex formed by the primer xtension. Once the strands are separated, the next step in PCR involves hybridizing the separated strands with prim rs that flank the target sequence. The primers are then extended to form complementary copies of the target strands. For successful PCR amplification, the primers are designed so that

the position at which each primer hybridizes along a duplex sequence is such that an extension product synthesized from one primer, when separated from the template (complement), serves as a template for the extension of the other primer. The cycle of denaturation, hybridization, and extension is repeated as many times as necessary to obtain the desired amount of amplified nucleic acid.

In the preferred embodiment of the PCR process, strand separation is achieved by heating the reaction to a sufficiently high temperature for a sufficient time to cause the denaturation of the duplex but not to cause an irreversible denaturation of the polymerase (see U.S. Patent No. 4,965,188). Template-dependent extension of primers in PCR is catalyzed by a polymerizing agent in the presence of adequate amounts of four deoxyribonucleoside triphosphates (typically dATP, dGTP, dCTP, and dTTP) in a reaction medium comprised of the appropriate salts, metal cations, and pH buffering system. Suitable polymerizing agents are enzymes known to catalyze template-dependent DNA synthesis. In the present invention, the initial template for primer extension is typically RNA. Reverse transcriptases (RTs) suitable for synthesizing a cDNA from the RNA template are well known. For example, *Thermus thermophilus (Tth)* DNA polymerase, a thermostable DNA polymerase with reverse transcriptase activity is marketed by Roche Molecular Systems (Alameda, CA).

PCR is most usually carried out as an automated process with a thermostable enzyme. In this process, the temperature of the reaction mixture is cycled through a denaturing region, a primer annealing region, and an extension reaction region automatically. Equipment specifically adapted for this purpose is commercially available from Roche Molecular Systems.

**2. Alternate amplificati n assays**

As described above, a preferred embodiment of the invention incorporates RT-PCR amplification. On of skill will recognize, however, that amplification of target sequences in a sample may be accomplished by any known method,

such as ligase chain reaction (LCR), Qβ-replicase amplification, transcription amplification, and self-sustained sequence replication, each of which provides sufficient amplification.

3. **Detection of amplification products**

As explained in detail below, the size of the amplified fragments (the "amplification products") produced by the methods of the invention is typically sufficient to distinguish polioviruses from either NPEV or poliovirus recombinants. Thus, in some embodiments of the invention, size fractionation (*e.g.*, gel electrophoresis) of the amplified fragments produced in a given sample can be used to distinguish poliovirus from other viruses of interest. This is typically carried out by amplifying a control containing known viruses (*e.g.*, isolated poliovirus) with the same primers used to amplify the sample of interest. After running the amplified sequences in an agarose gel and labeling with ethidium bromide according to well known techniques (*see*, Sambrook *et al.*), the pattern of bands in the sample and control are compared. The presence of different or additional bands in the sample as compared to the control, is an indication of the presence of NPEV or poliovirus recombinants.

Alternatively, the amplification products of the invention can be detected using oligonucleotide probes specific to the target nucleic acids. The probes are usually selected from regions of the genome of the NPEV or poliovirus that are specific to one or the other.

Sequence-specific probe hybridization is a well known method of detecting desired nucleic acids in a sample. Under sufficiently stringent hybridization conditions, the probes hybridize specifically only to substantially complementary sequences. The stringency of the hybridization conditions can be relax d to tolerate varying amounts of sequence mismatch. Detection of the amplified product utilizes this sequence-specific hybridization to insure detection of only the correct amplified target, thereby decreasing the chance of a false positive caused by the

presence of homologous sequences from related organisms or other contaminating sequences.

A number of hybridization formats well known in the art, including but not limited to, solution phase, solid phase, mixed phase, or *in situ* hybridization assays. In solution (or liquid) phase hybridizations, both the target nucleic acid and the probe or primer are free to interact in the reaction mixture. In solid phase hybridization assays, either the target or probes are linked to a solid support where they are available for hybridization with complementary nucleic acids in solution. Exemplary solid phase formats include Southern hybridizations, dot blots, and the like.

The hybridization complexes are detected according to well known techniques; such detection is not a critical aspect of the present invention. Nucleic acid probes capable of specifically hybridizing to a target can be labeled by any one of several methods typically used to detect the presence of hybridized nucleic acids. One common method of detection is the use of autoradiography using probes labeled with $^{3}H$, $^{125}I$, $^{35}S$, $^{14}C$, or $^{32}P$, or the like. The choice of radioactive isotope depends on research preferences due to ease of synthesis, stability, and half lives of the selected isotopes. Other labels include ligands which bind to antiligands or antibodies labeled with fluorophores, chemiluminescent agents, and enzymes. Alternatively, probes can be conjugated directly with labels such as fluorophores, chemiluminescent agents or enzymes. The choice of label depends on sensitivity required, ease of conjugation with the probe, stability requirements, and available instrumentation.

The probes and primers of the invention can be synthesized and labeled using well known techniques. Oligonucleotides for use as probes and primers may be chemically synthesized according to the solid phase phosphoramidite triester method first described by Beaucage, S.L. and Caruthers, M.H., 1981, Tetrahedron Letts., 22(20):1859-1862 using an automated synthesizer, as described in Needham-VanDevant r, D.R., *et al.* 1984, Nucleic Acids Res., 12:6159-6168. Purification of oligonucleotides is by either

native acrylamide gel lectrophoresis or by anion-exchange HPLC as described in Pearson, J.D. and Regnier, F.E., 1983, J. Chrom., 255:137-149.

### 4. Diagnosis of enteroviral conditions and diseases

The above described primers and assays are used to detect nonpolio enteroviruses in a sample, to serotype these viruses, to diagnose enteroviral diseases and medical conditions, and to correlate (or disprove a correlation between) specific symptoms or combinations of symptoms with the presence of a particular enterovirus. Diagnostic applications are supplemented and confirmed by an examination of the medical history and profile of the individual tested. Nonpolio enteroviral diseases, medical conditions and symptoms that are diagnosed by the methods of the invention encompass all diseases, medical conditions and symptoms reported to be associated with nonpolio enteroviruses here and in the scientific literature, specifically including aseptic meningitis, enteroviral diabetes mellitus, enteroviral conjunctivitis, acute flaccid paralysis, acute benign pericarditis, exanthema, enanthema, dilated cardiomyopathy, foot and mouth disease, chronic fatigue syndrome, febrile illnesses, and upper respiratory tract infections. The detection of nonpolio enteroviral infections and their correlation with medical conditions will make possible vaccines and methods of treatment.

### 5. Kits

The present invention also provide kits, multicontainer units comprising components useful for practicing the present method. A useful kit can contain probes for detecting the desired target nucleic acid, from either a recombinant virus or an NPEV. In some cases, the probes may be fixed to an appropriate support membrane. The kit will also contain primers provided in this invention. Other optional components of the kit include, for example, reverse-transcriptase or polymerase, the substrate nucleoside triphosphates, means used to label (for example, an avidin-

enzyme conjugate and enzyme substrate and chromogen if the label is biotin), and the appropriate buffers for reverse transcription, PCR, or hybridization reactions. In addition to the above components, the kit can also contain instructions for carrying out the method of this invention.

## EXAMPLES

### A. MATERIALS AND METHODS

**Viruses:**

Enterovirus isolates were identified by neutralization pools of immune sera (Melnick, *Virology*, pp. 549-605, 2nd ed., Raven Press, N.Y. (1990)) followed by confirmation of serotype with monotype neutralizing polyclonal antibodies. Viruses were propagated in HeLa or RD monolayers to produce high-titer inoculation stocks.

**Amino acid sequences:**

All of the VP1 amino acid sequences in Figure 2 were obtained from Genbank. Their accession numbers are as follows: CAV2-L28146, CAV9-D00627, CAV16-U05876, CAV21-D00538, CAV24-D90457, CBV1-M16560, CBV3-M33854, CBV4-X05690, CBV5-X67706, EV70-D00820, EV71-U22521, Echo 6-U05851, Echo 9-X84981, Echo 11-X80059, Echo 12-X77708. Brown and Fallansch (1995), *Virus Res*. 39:195-205.

**Oligonucleotide synthesis:**

Synthetic oligodeoxynucleotides were prepared, purified, and analyzed as described (Yang *et al*., *Virus Res*. *20*:159-179 (1991)). The degenerate primers used for virus amplification are listed in Table 1. Each NPEV, whose amino acid sequence in Fig. 1 provided the source of the targeted amino acids, is identified along with the numbers in parentheses indicating the genomic intervals matching these amino acids.

**PCR amplification and analysis:**

*In vitro* amplification by PCR was performed as described previously (Kilpatrick *et al*., *J. Clin. Micro*. (Dec.

1996)). Amplification reactions were carried out in 50 μl reaction mixtures containing 1 μl of each individual virus tissue culture lysate in 50 mM Tris-HCl (pH 8.3), 70 mM KCl, 5 mM $MgCl_2$, 10 mM dithiothreitol, 80 pmol of each degenerate primer, 200 μM each of dATP, dCTP, dGTP, dTTP (Pharmacia), 0.5% NP-40, 5 U placenta ribonuclease inhibitor (Boehringer Mannheim Biochemicals, Indianapolis, IN), 1.25 U AMV reverse transcriptase (Boehringer Mannheim), and 1.25 U of Taq DNA polymerase (Perkin Elmer- Cetus, Norwalk, CT). The reaction mixtures were prepared, excluding the ribonuclease inhibitor, Amv reverse transcriptase, and Taq DNA polymerase, overlaid with mineral oil, heated for 5 min at 95°C to release the virion RNA and chilled on ice. The enzymes were then added and the samples incubated at 42°C for 30 min before 30 cycles of programmed amplification (denaturation:94°C, 1 min; annealing: 42°C, 1 min; extension: 60°C, 1 min) in a DNA thermal cycler (Perkin Elmer-Cetus). Conditions for polyacrylamide gel electrophoresis, and detection of amplified products by ethidium bromide staining were as described (Yang *et al., Virus Res. 20*:159-179 (1991)).

**Selection of primer binding sites.**

The VP1 sequence information for 15 prototype NPEVs is shown in Figure 2. VP1 sequences for the approximately 50 remaining NPEVs have not been determined.

Several areas of amino acid conservation were identified in Figure 2. The conserved sequences were then used to generate a series of primers which could be used in amplification reactions to detect enteroviral serotypes. A representative list of peptide target sequences and primer sequences is depicted in Table 1.

## TABLE 1: NPEV PCR PRIMERS

| PRIMER | TARGETED PEPTIDE SEQUENCE | DEGENERATE PRIMER SEQUENCE |
|---|---|---|
| 1A | FGQQSGA (3-9)[2-CBV-1] | 5'-GCICCIGAYTGITGICCRAA |
| 5S | MYVPPGG (142-148)[1] | 5'-ATGTAYGTICCICCIGGIGG |
| 6A | WTEGNAP (169-175)[1] | 5'-GGIGCRTTICCYTCIGTCCA |
| 6S | WTEGNAP (169-175)[1] | 5'-TGGACIGARGGIAAYGCICC |
| 7A | N(ts)LNNM (208-213)[1] | 5'-CATRTTRTTIARIGWITT |
| 7S | N(ts)LNNM (208-213)[1] | 5'-AAIWCIYTIAAYAAYATG |
| 8A | GATG(yq)QS (1-7)[2-CBV-1] | 5'-GATTGSTIICCRAAIGCKCC |
| 9A | FKPKHVK (237-243)[1] | 5'-TTIACRTGYTTIGGYTTRAA |
| 11A | TMQTRHV (47-53)[1] | 5'-ACRTGICIIGTYTGCATIGT |
| 14S | A(mi)(gv)RVAD (10-16)[1] | 5'-GCIATIGKIMGIGTIGCIGA |
| 24S | PALTA(av)E (42-48)[1] | 5'-CCIGCICTYACTGCIGYKG |
| 25A | NY(kh)(st)RSE (63-69)[3] | 5'-TCAGAICIIGWITKRTARTT |
| 27A | PALTAVE (42-48)[1] | 5'-TCCACIGCAGTIAGWGCWGG |
| 28A | GEVRNQ (143-148)[3] | 5'-CARGTICGIACYTCCCC |
| 33A | QNQDAQI | 5'-ATITGIGCITCYTGRTTYTG |
| 34S | FTYVRFD (107-113)[4] | 5'-TTIACITAYGTICGITTYGA |
| 35A | PVQT(hq)QI (135-141)[1] | 5'-ATYTGITGIGTYTGIACWGG |
| 36S | ELTFVIT (115-121)[1] | 5'-GARYTIACITTYGTIATAAC |
| 38A | MPVLTRQ (73-79)[5] | 5'-TGICGIGTYAAIACIGGCAT |
| 39S | FTYMRFD (107-113)[6] | 5'-TTIACITAYATGCGITTYGA |
| 40A | NGELVPQ (143-149)[6] | 5'-TGIGGIACIAGYTCICCRTT |
| 41A | CTPTGRV (140-146)[7] | 5'-ACYCTICCIGTIGGKGTRCA |
| 43A | MY(vi)P(tp)GA (153-159)[3] | 5'-GCICCIGKIGGIAYRTACAT |
| 46A | NYHSRSE (55-61)[1] | 5'-TCIGAICTIGWI[illegible]GRTARTT |
| 47A | MQTRHV(kh) (48-54)[1] | 5'-TKIACRTGICKIGTYTGCAT |
| 51S | (cnm)FYDGW (191-196)[1] | 5'-AWITTYTAYGAYGGITGG |
| 52A | NNMGT(il)Y (211-217)[1] | 5'-TAIAIIGTICCCATRTTRTT |
| 54A | NNNYVGQ (255-261![8] | 5'-TGICCIACRTAITTRTTRTT |
| 55S | VVNSYQP (215-22I)[8] | 5'-GTIGTIAAYTSITAYCARCC |
| 59S | GDGIADM (1-7)[6] | 5'-GGIGAYGGIATIGCIGAYATG |
| 61S | MYVPGGA (153-159)[3] | 5'-ATGTAYRTICCIMCIGGIGC |
| 62S | IDQTVNN | 5'-ATIGAYCAYACIGTIAAYAA |
| 63S | ITERYYT (140-146)[9] | 5'-ATIACIGARIGITAYTAYAC |
| 64S | DENLIET (60-66)[6] | 5'-GAIGARAAYCTIATIGARAC |
| 65A | WDID(il)(mt)G (109-115)[6] | 5'-CCCATIAKRTCIATRTCCC |
| 67S | KHV(rk)AWV (140-146)[1] | 5'-AARCAYGTIARIGCITGGGT |
| 68A | K(lm)TDPPP (182-188)[1] | 5'-GGIGGIGGRTCIGTIAKYTT |
| 69A | MGYAQ(ml)R (114-120)[6] | 5'-CGIAKYTGIGCRTAICCCAT |
| 73A | D(tm)PVLTH (136-142)[10] | 5'-TGIGTIAGIACIGGCRTRTC |
| 74A | FYDGFA (203-208)[1] | 5'-GCIAAICCRTCRTARAA |
| 76A | WQTATNP (181-187)[6] | 5'-GGRTTIGTIGCIGTYTGCCA |
| 77A | MFVPPGA (164-160)[7] | 5'GCICCIGGIGGIACRWACAT |
| 78A | DWQ (rn) CVW (30-36)[2-CBV-B3] | 5'-CCCAIACRCAIITYTGCCARTC |
| 79A | NRDLLVS (37-43) [2-CAV-9] | 5'-CTYACIAIIAGRTCYCTRTT |
| 80A | RDLLVST (38-44)[2-ECH-12] | 5'-GTRCTYACIAIIAGRTCYCT |
| 81A | AQGSDNI (45-51)[2-CAV-24] | 5'-ATIGTRTCISICCCYTGSGC |
| 82A | GKFGQQS (1-6)[2-CAV-16] | 5'-GAITGYTGICCRAAYTTTCC |
| 83A | GAFGYQS (1-6)[2-ECH-11] | 5'-GATTGSTIICCRAAIGCKCC |
| 84A | GRFG(hq)Q (3-9)[2-CAV-2] | 5'-CTGKTGICCRAAICTSCC |

* A = antisense, S = sense

** All amino acid residues (with c rresponding position numbers) are located in VP1, with the exception f #2- which is located in the 2A nonstructural protein and are from the following isolates: 1 = CBV-B1; 2 = CBV-B1; 3 = CAV-A21; 4 = CBV-B3; 5 = CAV-A9; 6 = CAV-A16; 7 = EV71; 8 = EV70; 9 = CAV-A24; 10 = echovirus 12; 11 = Rhinovirus II

*** (Mixed base residues are as follows: Y = both T and C; R = A and G; M = A & C; K = G & T; S = G & C; W = A & T; I = deoxyinosine)

To complement all possible codon combinations the selected primer sit s, the primers of the present invention contain either mixed-base residues or, preferably, deoxyinosine residues at degenerate codon positions. Deoxyinosine residues, which can pair with all four bases (Martin *et al.*, *Nucleic Acids Res.* *13*:8927-8938 (1985); Ohtsuka *et al.*, *J. Biol. Chem.* *260*:2605-2608 (1985); Rossolini *et al.*, *Mol. Cell. Probes* *8*:91-98 (1994)) were incorporated into the primers to match those positions having possible four-fold degeneracy.

The following is a partial list of possible primer pairs:
5S/6A (SEQ ID NO:49/SEQ ID NO:50),
5S/74A (SEQ ID NO:49/SEQ ID NO:87),
6S/7A (SEQ ID NO:51/SEQ ID NO:52),
7S/8A (SEQ ID NO:53/SEQ ID NO:54),
7S/9A (SEQ ID NO:53/SEQ ID NO:55),
14S/11A (SEQ ID NO:57/SEQ ID NO:56),
24S/47A (SEQ ID NO:58/SEQ ID NO:72),
24S/25A (SEQ ID NO:58/SEQ ID NO:59),
24S/46A (SEQ ID NO:58/SEQ ID NO:71),
34S/28A (SEQ ID NO:63/SEQ ID NO:61),
34S/33A (SEQ ID NO:63/SEQ ID NO:62),
34S/35A (SEQ ID NO:63/SEQ ID NO:64),
34S/38A (SEQ ID NO:63/SEQ ID NO:66),
34S/73A (SEQ ID NO:63/SEQ ID NO:86),
36S/35A (SEQ ID NO:65/SEQ ID NO:64),
39S/40A (SEQ ID NO:67/SEQ ID NO:68),
39S/41A (SEQ ID NO:67/SEQ ID NO:69),
51S/52A (SEQ ID NO:73/SEQ ID NO:74),
55S/54A (SEQ ID NO:76/SEQ ID NO:75),
59S/27A (SEQ ID NO:77/SEQ ID NO:60),
61S/68A (SEQ ID NO:78/SEQ ID NO:84),
62S/27A (SEQ ID NO:79/SEQ ID NO:60),
63S/43A (SEQ ID NO:80/SEQ ID NO:70),
64S/69A (SEQ ID NO:81/SEQ ID NO:85),
64S/65A (SEQ ID NO:81/SEQ ID NO:82),
67S/1A (SEQ ID NO:83/SEQ ID NO:48), and
67S/8A (SEQ ID NO:83/SEQ ID NO:54).

One example of how the information of Figure 2 was used to sel ct primer pairs is shown by the degenerate primer pair 5S/6A. The sense primer 5S targets the amino acids MYVPPGG (a.a. # 142-148 in CBV1, for example). This amino acid sequence is highly conserved in all known NPEVS. In addition, Palmenburg, *Molecular Aspects of Picornavirus Infection and Detection*, pp. 215-230, American Society of Microbiology (1989) showed this amino acid sequence is highly conserved among polioviruses and rhinoviruses as well. The antisense primer 6A is the selective primer in the amplification reaction and recognizes the amino acids WTEGNAP (a.a. # 169-175 in CBVI, for example). This primer is uniquely conserved primarily among CBVs and those NPEVs closely related to CBVS, such as CAV9, and all four sequenced echoviruses (Figure 2).

The fact that the 5S/6A primer set also appears to recognize CAV9 is not unexpected. Other researchers have shown, using sequence alignment programs, that CAV9 is very closely related to CBVs (Pulli *et al.*, *Virology*, in press (1995)). Many other conserved amino acid epitopes (6-7 residues in length) were identified and are listed in Table 1 along with their relative position within VPI and their corresponding degenerate PCR primer.

Some primers were designed to recognize more than 1 amino acid at a particular residue and are indicated by parentheses around that residue in Table 1. For example, primer 7S (which codes for the peptide sequence N(ts)LNNM) was designed to recognize nucleotides which code for either a threonine residue (found in CBVI) or a serine residue (found in CBV4) in the second amino acid position of the primer. As a result, the synthesis of this primer results in several species of 23 primers with one-half of the primer species containing TGI residues (which encodes for Serine) and the other one-half containing AGI residues (which encodes for Threonine) in positions 4, 5, and 6, respectively.

Some of the other group-specific primer pairs include 51S/52A, which recognizes all known CBV isolates. In this cas , the sense primer 51S is the selective primer in the amplification reaction with the "FYDGW" amino acid sequence

being specific for CBVs and closely related serotypes such as many of the echoviruses, while excluding CAVs from amplification. On the other hand, primers 61S/68A and 64S/65A were designed using CAV16 known sequences to target CAVs in general.

**B. Amplification of enterovirus templates.**

In order to determine if all of the isolates tested in this report contain amplifiable viral templates, these isolates were first tested with PCR primers (data not shown) which recognize all enteroviruses (Yang *et al.*, *Virus Res.* *20*:159-179 (1991)). Each of the primers listed in Table 1 were initially tested on a panel of viruses representing major NPEV groups (including CAV9, 12, 21, CBV1-6, echoviruses 4, 11, 30, and EV71). The results from screening this virus panel, shown in Fig 3, were used to determine how conserved the targeted amino acid sites were among this selected virus group.

Sequence analysis of the PCR products amplified from templates of several viruses (for example the 101 bp amplification product from the 5S/6A primer set) confirmed that the primers had primed specific amplification of the targeted nucleotide interval (data not shown). General patterns of reactivity can be determined from these results. In particular, primer sets 5S/6A, 7S/9A, 14S/11A, and 51S/52A were found to be broadly reactive with both CBVs and echoviruses. This amplification pattern agrees with the method, discussed above, that was used to select these particular primer sites. The amino acids for these four primer sets are shown to be conserved throughout the CBVs as well as the echoviruses in Fig. 2.

The four antisense primer sequences (*i.e.* primers 6A, 9A, 11A and 52A) are not seen in the known CAV sequences shown in Fig. 2. This explains why there is no amplification when these primers are used on CAV isolates (with the noted exception of CAV9).

Many of the remaining primers tested in Fig 3 reacted with either a few virus isolates, or none at all. Primer sets

that were either broadly reactive or s lective in amplification were further test d using a larger panel of prototype NPEVS.

**C. NPEV PCR primer pool.**

A collection of 49 NPEVs was assembled for further PCR analysis. The bulk of NPEVs not found in this collection consists of CAVs, which grow very poorly in cell culture (Muir *et al.*, *J. Clin. Micro.* *31*:31-38 (1993); Rotbart, *et al.*, *Human Enterovirus Infections*, pp. 401-418 (1995)).

This expanded virus panel was tested with PCR primer sets identified in Fig. 3. Primers 5S/6A, 7S/9A, 14S/11A and 51S/52A were found to be broadly reactive through all CBVs and most of the echoviruses (Figure 3). Primer set 67S/SA was shown to amplify selected echoviruses (EC11, 12, 17, 19, 21, 24, 29, and 31) without reacting with CAVs and CBVs. The 67S/1A primer set reacted with a few CAVs (A3 & A8) and several echoviruses (EC9, 11, 12, 13, 17, 21, 24, 29, and 31). EV69 was also amplified by 67S/1A. The primer sets 61S/68A and 64S/65A amplified the predicted PCR product size with only CAVs (A3, A4, A5, A6, A8, A10, A16 for 61S/68A and A8, A10, A14, A16 for 64S/65A), as well as those viruses closely related to CAVs, *i.e.* EV70 and EV71. The 8 PCR primer sets identified in Fig. 3 can be used as a collection or "pool" of primer sets for rapidly performing a preliminary screen against suspected NPEV isolates.

A quick screen chart was assembled using these primers to aid in the screening of NPEVs (Figure 4). This chart also shows that none of these primers reacted with CAV12, 21, 24 and did not detect EV68. Other PCR results shown in Fig. 1 specifically detect either CAV12 (39S/40A) or CAV21 (34S/28A). Primers, 63S/43A, specifically detect CAV24 (data not shown). Only one NPEV, EV68, was not amplified by PCR primers in this report. Twelve of the isolates can be specifically id ntified using this prim r pool (*i.e.* CAV3, 8, 14, EC9, 11, 13, 14, 17, 19, 24, 31, and EV69). However, until an extensive sequence database for all prototype NPEVs can be assembled by sequencing the PCR pr ducts in this report, all virus isolates yielding positive PCR reactions (*i.e.*,

amplification products of the corr ct size for each primer set), should be serotyped using monospecific antisera in micro-neutralization tests, if at all possible. By first using these PCR primers to quickly screen virus isolates (within 1 day), one can concentrate on performing micro-neutralization tests with only those monospecific antisera suggested by the PCR results.

PCR assays using the degenerate panPV/PCR primers were positive for a very diverse sample of poliovirus genotypes, had excellent diagnostic specificities, and had template sensitivities comparable to those obtained with non-degenerate primers. Similar PCR primer designs should be directly applicable to the detection of NPEVS.

Amino acid sequences seen in a particular group or serotype can be specifically targeted using degenerate PCR primers, providing that the targeted amino acids are truly unique to that group or serotype. Either sense or antisense primers can provide the selectivity, with the remaining primer in the reaction having a broader reactivity to other viruses not in the targeted group.

Since NPEVs consist of positive sense, single strand RNA, it is preferred to have an antisense selective primer since this primer will be responsible for the initial CDNA synthesis, thus initially amplifying only the targeted sequences. The majority of primers in Table 1 which were used for specific template amplifications use the antisense primer for the selectivity of the reaction, such as the 6A, 9A, 11A and 52A primers which have a broad reactivity against CBvs and echoviruses. However, several sense-polarity primers are also used for specific selection.

For example, the sense primer 59S targets the amino acid sequence unique to CAV16 (GDGIADM) and therefore amplifies only CAV16 despite the fact that the antisense primer 27A (which is complementary to the sequ nce that encodes the p ptide PALTAVE) targets a widely conserv d site found in almost all enteroviruses. In another case, the sense primer 63S targets the amino acids unique to CAV24 (ITERYYT) and therefore amplifies only CAV24, ven though the 43A primer

(MYVPPPGA) in this set targets an epitope that is widely conserved among all enteroviruses. The identification of such conserved amino acid epitopes allowed us to design specific PCR primers that could identify 97% of the NPEVs in our collection.

Using these primers sets together (as shown in Figure 5) for preliminary screening provides a powerful tool in identifying NPEVS. The use of PCR to perform preliminary screens for NPEVS should speed the identification of virus isolates by reducing the numbers of micro-neutralization assays that need to be performed. Also, PCR with degenerate primers has been shown to detect as little as 100 fg of polivovirus RNA (Kilpatrick, *et al.*, *J. Clin. Micro.* (published in Dec 1996)). Similar sensitivities should be expected for detecting NPEV serotypes.

The RNAs from 48 out of a total 49 different prototype NPEVs used for testing were detected by PCR. Even though the nucleic acid sequences within VPI for the majority of these NPEVs (35/49) were unknown prior to testing, these isolates were detected due to the high amino acid conservation in the targeted epitopes.

Now that specific PCR primers have been identified which can amplify within the VP1 gene of almost all NPEVs (48/49 tested), sequence databases can be established which will yield even more type-specific sequences. These nucleotide sequences will be the targets for even more specific molecular reagents (*i.e.* primers and probes) which will further increase the speed, efficiency, and accuracy of future NPEV identification.

All publications, patents and patent applications mentioned in this specification are hereby incorporated by reference for all purposes into the specification to the same extent as if each individual publication, patent or patent application had been specifically and individually indicated to be incorporated by r f r nce. Although the foregoing invention has b en described in some d tail by way of illustration and example for purposes of clarity of understanding, it will be obvious that certain changes and

modifications may be practiced within the scope of the appended claims. For instance, primers that sp cifically hybridize to 5' nontranslated region of an enteroviral genome or to other enteroviral proteins may be used. In another embodiment, the present invention contemplates assays wherein the primer pairs disclosed above are combined with primer pairs that recognize other viral species. For example, patent publication No. 95/02704, and U.S. Patent applications Serial Nos. 08/092,110 and 08/273,474 (incorporated by reference herein) describe primers that specifically detect polioviruses. In addition, primers 85A and 86S target known rhinoviruses (which are also in the Picornavirus family) and would be very useful in differentiating between upper respiratory infections that are caused by rhinoviruses, and repiratory infections caused by members of the enterovirus group.

| PRIMER | TARGETED PEPTIDE SEQUENCE | DEGENERATE PRIMER SEQUENCE |
|---|---|---|
| 85A | QPED(av)IE (46-52)$^{\text{II-RIII-2}}$ | 5'-TCRATIITRTCYTCIGGYTG |
| 86S | NPVE(nh)YI (1-7)$^{\text{II-RIII-2}}$ | 5'-AAICCIGTYGARIAYTAYAT |

WHAT IS CLAIMED IS:

1. A pair of oligonucleotide primers which are used to detect the presence or absence of a nonpolio enterovirus in a sample and to identify nonpolio enterovirus serotypes, wherein:

a first primer of said pair hybridizes specifically to a sense strand of a first nonpolio enterovirus nucleic acid sequence that encodes a first conserved nonpolio enteroviral peptide sequence, and

a second primer of said pair hybridizes specifically to an antisense strand of a second nonpolio enterovirus nucleic acid sequence that encodes a second conserved nonpolio enteroviral peptide sequence, wherein both conserved sequences are from a same nonpolio enteroviral protein,

wherein said pair of primers in an amplification protocol yields an amplification product of a nonpolio enteroviral sequence that encodes both the first and second conserved nonpolio enteroviral peptide sequence.

2. A pair of primers according to claim 1, wherein the primers are degenerate and contain deoxyinosine.

3. A pair of primers according to claim 1, wherein the nonpolio enteroviral protein is VP1.

4. A pair of primers according to claim 1, wherein the conserved peptide sequence of an enterovirus protein is selected from the group of sequences consisting essentially of:

SEQ ID NO:1: FGQQSGA,
SEQ ID NO:2: MYVPPGG,
SEQ ID NO:3: WTEGNAP,
SEQ ID NO:4: N(ts)LNNM,
SEQ ID NO:5: GATG(yq)QS,
SEQ ID NO:6: FKPKHVK,
SEQ ID NO:7: TMQTRHV,
SEQ ID NO:8: A(mi)(gv)RVAD,
SEQ ID NO:9: PALTA(av)E,

SEQ ID NO:10: NY(kh)(st)RSE,
SEQ ID NO:11: PALTAVE,
SEQ ID NO:12: GEVRNQ,
SEQ ID NO:13: QNQDAQI
SEQ ID NO:14: FTYVRFD,
SEQ ID NO:15: PVQT(hq)QI,
SEQ ID NO:16: ELTFVIT,
SEQ ID NO:17: MPVLTRQ,
SEQ ID NO:18: FTYMRFD,
SEQ ID NO:19: NGELVPQ,
SEQ ID NO:20: CTPTGRV,
SEQ ID NO:21: MY(vi)P(tp)GA,
SEQ ID NO:22: NYHSRSE,
SEQ ID NO:23: MQTRHV(kh),
SEQ ID NO:24: (cnm)FYDGW,
SEQ ID NO:25: NNMGT(il)Y,
SEQ ID NO:26: NNNYVGQ,
SEQ ID NO:27: VVNSYQP,
SEQ ID NO:28: GDGIADM,
SEQ ID NO:29: MYVPGGA,
SEQ ID NO:30 IDQTVNN
SEQ ID NO:31: ITERYYT,
SEQ ID NO:32: DENLIET,
SEQ ID NO:33: WDID(il)(mt)G,
SEQ ID NO:34: KHV(rk)AWV,
SEQ ID NO:35: K(lm)TDPPP,
SEQ ID NO:36: MGYAQ(ml)R,
SEQ ID NO:37: D(tm)PVLTH,
SEQ ID NO:38: FYDGFA,
SEQ ID NO:39: WQTATNP,
SEQ ID NO:40: MFVPPGA,
SEQ ID NO:41: DWQ(rn)CVW,
SEQ ID NO:42: NRDLLVS,
SEQ ID NO:43: RDLLVST,
SEQ ID NO:44: AQGSDNI,
SEQ ID NO:45: GKFGQQS,
SEQ ID NO:46: GAFGYQS, and
SEQ ID NO:47: GRFG(hq)Q.

5. A pair of primers according to claim 4, consisting of an RDLLVST oligonucleotide having a sequence selected from the group of sequences consisting essentially of:

SEQ ID NO:48: 5'-GCICCIGAYTGITGICCRAA,
SEQ ID NO:49 5'-ATGTAYGTICCICCIGGIGG,
SEQ ID NO:50: 5'-GGIGCRTTICCYTCIGTCCA,
SEQ ID NO:51: 5'-TGGACIGARGGIAAYGCICC,
SEQ ID NO:52: 5'-CATRTTRTTIARIGWITT,
SEQ ID NO:53: 5'-AAIWCIYTIAAYAAYATG,
SEQ ID NO:54: 5'-GATTGSTIICCRAAIGCKCC,
SEQ ID NO:55: 5'-TTIACRTGYTTIGGYTTRAA,
SEQ ID NO:56: 5'-ACRTGICIIGTYTGCATIGT,
SEQ ID NO:57: 5'-GCIATIGKIMGIGTIGCIGA,
SEQ ID NO:58: 5'-CCIGCICTYACTGCIGYKG,
SEQ ID NO:59: 5'-TCAGAICIIGWITKRTARTT,
SEQ ID NO:60: 5'-TCCACIGCAGTIAGWGCWGG,
SEQ ID NO:61: 5'-CARGTICGIACYTCCCC,
SEQ ID NO:62: 5'-ATITGIGCITCYTGRTTYTG,
SEQ ID NO:63: 5'-TTIACITAYGTICGITTYGA,
SEQ ID NO:64: 5'-ATYTGITGIGTYTGIACWGG,
SEQ ID NO:65: 5'-GARYTIACITTYGTIATAAC,
SEQ ID NO:66: 5'-TGICGIGTYAAIACIGGCAT,
SEQ ID NO:67: 5'-TTIACITAYATGCGITTYGA,
SEQ ID NO:68: 5'-TGIGGIACIAGYTCICCRTT,
SEQ ID NO:69: 5'-ACYCTICCIGTIGGKGTRCA,
SEQ ID NO:70: 5'-GCICCIGKIGGIAYRTACAT,
SEQ ID NO:71: 5'-TCIGAICTIGWRTGRTARTT,
SEQ ID NO:72: 5'-TKIACRTGICKIGTYTGCAT,
SEQ ID NO:73: 5'-AWITTYTAYGAYGGITGG,
SEQ ID NO:74: 5'-TAIAIIGTICCCATRTTRTT,
SEQ ID NO:75: 5'-TGICCIACRTAITTRTTRTT,
SEQ ID NO:76: 5'-GTIGTIAAYTSITAYCARCC,
SEQ ID NO:77: 5'-GGIGAYGGIAT1GCIGAYATG,
SEQ ID NO:78: 5'-ATGTAYRTICCIMCIGGIGC,
SEQ ID NO:79: 5'-ATIGAYCAYACIGTIAAYAA
SEQ ID NO:80: 5'-ATIACIGARIGITAYTAYAC,
SEQ ID NO:81: 5'-GAIGARAAYCTIATIGARAC,
SEQ ID NO:82: 5'-CCCATIAKRTCIATRTCCC,

SEQ ID NO:83: 5'-AARCAYGTIARIGCITGGGT,
SEQ ID NO:84: 5'-GGIGGIGGRTCIGTIAKYTT,
SEQ ID NO:85: 5'-CGIAKYTGIGCRTAICCCAT,
SEQ ID NO:86: 5'-TGIGTIAGIACIGGCRTRTC,
SEQ ID NO:87: 5'-GCIAAICCRTCRTARAA,
SEQ ID NO:88: 5'-GGRTTIGTIGCIGTYTGCCA,
SEQ ID NO:89: 5'-GCICCIGGIGGIACRWACAT,
SEQ ID NO:90: 5'-CCCAIACRCAIITYTGCCARTC,
SEQ ID NO:91: 5'-CTYACIAIIAGRTCYCTRTT,
SEQ ID NO:92: 5'-GTRCTYACIAIIAGRTCYCT,
SEQ ID NO:93: 5'-ATIGTRTCISICCCYTGSGC,
SEQ ID NO:94: 5'-GAITGYTGICCRAAYTTTCC,
SEQ ID NO:95: 5'-GATTGSTIICCRAAIGCKCC, and
SEQ ID NO:96: 5'-CTGKTGICCRAAICTSCC.

6. A pair of primers according to claim 1, selected from the group of primer pairs consisting of:
5S/6A (SEQ ID NO:49/SEQ ID NO:50),
5S/74A (SEQ ID NO:49/SEQ ID NO:87),
6S/7A (SEQ ID NO:51/SEQ ID NO:52),
7S/8A (SEQ ID NO:53/SEQ ID NO:54),
7S/9A (SEQ ID NO:53/SEQ ID NO:55),
14S/11A (SEQ ID NO:57/SEQ ID NO:56),
24S/47A (SEQ ID NO:58/SEQ ID NO:72),
24S/25A (SEQ ID NO:58/SEQ ID NO:59),
24S/46A (SEQ ID NO:58/SEQ ID NO:71),
34S/28A (SEQ ID NO:63/SEQ ID NO:61),
34S/33A (SEQ ID NO:63/SEQ ID NO:62),
34S/35A (SEQ ID NO:63/SEQ ID NO:64),
34S/38A (SEQ ID NO:63/SEQ ID NO:66),
34S/73A (SEQ ID NO:63/SEQ ID NO:86),
36S/35A (SEQ ID NO:65/SEQ ID NO:64),
39S/40A (SEQ ID NO:67/SEQ ID NO:68),
39S/41A (SEQ ID NO:67/SEQ ID NO:69),
51S/52A (SEQ ID NO:73/SEQ ID NO:74),
55S/54A (SEQ ID NO:76/SEQ ID NO:75),
59S/27A (SEQ ID NO:77/SEQ ID NO:60),
61S/68A (SEQ ID NO:78/SEQ ID NO:84),

62S/27A (SEQ ID NO:79/SEQ ID NO:60),
63S/43A (SEQ ID NO:80/SEQ ID NO:70),
64S/69A (SEQ ID NO:81/SEQ ID NO:85),
64S/65A (SEQ ID NO:81/SEQ ID NO:82),
67S/1A (SEQ ID NO:83/SEQ ID NO:48), and
67S/8A (SEQ ID NO:83/SEQ ID NO:54).

7. A collection of primer pairs according to claim 1, consisting of the following eight primer pairs:
5S/6A (SEQ ID NO:49/SEQ ID NO:50),
7S/9A (SEQ ID NO:53/SEQ ID NO:55),
14S/11A (SEQ ID NO:57/SEQ ID NO:56),
51S/52A (SEQ ID NO:73/SEQ ID NO:74),
61S/68A (SEQ ID NO:78/SEQ ID NO:84),
64S/65A (SEQ ID NO:81/SEQ ID NO:82),
67S/1A (SEQ ID NO:83/SEQ ID NO:48), and
67S/8A (SEQ ID NO:83/SEQ ID NO:54).

8. An assay for detecting the presence or absence of a nonpolio enterovirus nucleic acid sequence in a sample, comprising:

(a) contacting the sample with a first pair of oligonucleotide primers according to claim 1 in an amplification protocol;

(b) contacting the sample with a second pair of oligonucleotide primers according to claim 1, different from those used in step (a), in an amplification protocol; then

(c) determining the presence or absence of a nonpolio enterovirus by detecting for the presence or absence of amplification products.

9. An assay according to claim 8, wherein steps (a) and (b) are repeated with a third or more pairs of oligonucleotide primers according to claim 1, but different from the first or second pair.

10. An assay according to claim 8, further wherein the amplification products are compared to determine th presence or absence of a serotype of a nonpolio enterovirus.

11. An assay according to claim 11, wherein the primer pairs are:
5S/6A (SEQ ID NO:49/SEQ ID NO:50),
7S/9A (SEQ ID NO:53/SEQ ID NO:55),
14S/11A (SEQ ID NO:57/SEQ ID NO:56),
51S/52A (SEQ ID NO:73/SEQ ID NO:74),
61S/68A (SEQ ID NO:78/SEQ ID NO:84),
64S/65A (SEQ ID NO:81/SEQ ID NO:82),
67S/1A (SEQ ID NO:83/SEQ ID NO:48), and
67S/8A (SEQ ID NO:83/SEQ ID NO:54).

12. An assay according to claim 8, wherein the sample is a biological fluid.

13. An assay according to claim 8, wherein the assay is diagnostic for a disease caused by a non-polio enterovirus.

14. An assay according to claim 14, wherein the disease is a member of the group that consists of aseptic meningitis, enteroviral diabetes mellitus, enteroviral conjunctivitis, acute flaccid paralysis, acute benign pericarditis, exanthema, enanthema, dilated cardiomyopathy, foot and mouth disease, chronic fatigue syndrome, febrile illnesses, and upper respiratory tract infections.

15. A kit for detecting a non-polio enterovirus in a sample, comprising:

at least two pairs of primers according to claim 1, and

instructions for using the kit in an assay to amplify DNA using the primers.

16. A kit according to claim 16, wherein the kit includes at least eight pairs of primers.

17. A kit according to claim 17, wherein the primer pairs are:

5S/6A (SEQ ID NO:49/SEQ ID NO:50),
7S/9A (SEQ ID NO:53/SEQ ID NO:55),
14S/11A (SEQ ID NO:57/SEQ ID NO:56),
51S/52A (SEQ ID NO:73/SEQ ID NO:74),
61S/68A (SEQ ID NO:78/SEQ ID NO:84),
64S/65A (SEQ ID NO:81/SEQ ID NO:82),
67S/1A (SEQ ID NO:83/SEQ ID NO:48), and
67S/8A (SEQ ID NO:83/SEQ ID NO:54).

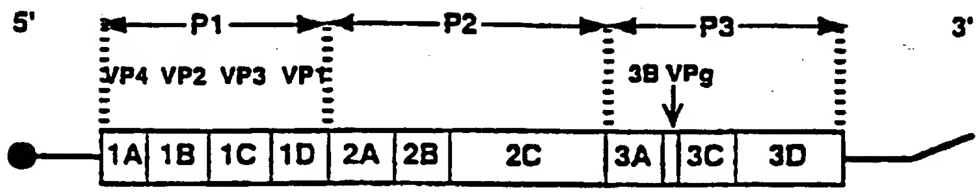


FIGURE 1

# NPEV VP1 AMINO ACID ALIGNMENT

A)

```
CAV24    GIEETIDTVISNALQLSQPKPQKQLTAQSTPSTS....GVNSQEVPALTAVETGVSGQAIPSDVIETRHVVNYKTRSESTL
CAV21    GIEDLIDTAIKNALRVSQPLRPSQLKQ.........PNGVNSQEVPALTAVETGASGQAIPSDVVETRHVINYKTRSESCL
CAV16    GDGIADMIDQAVTSRVGRALTSLQVEPTAANTNASE.HRLGTGLVPALQAAETGASSNAQDENLIETRCVLNHHSTQETTI
CAV2     GDGIEDAITNTVNATINRVLDRPISHSSTAANTQVSQHSIETGRVPALQAAETGATSNASDENLIETRCVVNKNSVEEASL
CAV9     GDVEEAIERARCTVADTMRTGPS.................NSASVPALTAVETGHTSQVTPSDTMQTRHVKNYHSRSESTV
CBV1     GPVEESVERAMVRVADTVSSKPT.................NSESIPALTAAETGHTSQVVPSDTMQTRHVKNYHSRSESSI
CBV3     GPVEDAITAAIGRVADTVGTGPT.................NSEAIPALTAAETGHTSQVVPGDTMQTRHVKNYHSRSESTI
CBV4     GPTEESVERAMGRVADTIARGPS.................NSEQIPALTAVETGHTSQVDPSDTMQTRHVHNYHSRSESSI
CBV5     GPPGEAVERAIARVADTISSGPV.................NSESIPALTAAETGHTSQVVPADTMQTRHVKNYHSRSESTV
Echo6    NDVQNAVERSIVRVADTLPSGPS.................NSESIPALTAAETGHTSQVVPSDTIQTRHVRNFHVRSESSV
Echo9    SDVREAVEGAIGRVADTIRSGPS.................NSEAVPALTAAETGHTSQVVPSDTMQTRHVKNYHSRSESTI
Echo11   GDVVEAVENAVARVADTIGSGPS.................NSQAVPALTAVETGHTSQVTPSDTMQTRHVKNYHSRSESSI
Echo12   GDVEEAVNRAVARVADTLPTGPR.................NSESIPALTAAETGHTSQVVPGDTMQTRHVKNYHSRTESSV
EV70     GEIVKTVANTVE.........................SEIKAELGVIPSLNAVETGATSNTEPEEAIQTRTVINMHGTAECLV
EV71     GDRVADVIESSIGDSVSRALTQALPAPTGQNTQVS.SHRLDTGEVPALQAAEIGASSNTSDESMIETRCVLNSHSTAETTL

CAV24    ESFFGRSACVTMLEVENFNATTEADKKKQFTTWAITYTDTVQL.RRKLEFFTYSRFDLEMTFVITERYYTSNT..GYARNQVYQ
CAV21    ESFFGRAACVTILSLTNSSKSGEE..KKHFNIWNITYTDTVQL.RRKLEFFTYSRFDLEMTFVFTENYPSTAS..GEVRNQCDQ
CAV16    GNFFSRAGLVSIITMPTTGTQNTDGY....VNWDIDLMGYAQM.RRKCELFTYMRFDAEFTFVAAKPN.......GELVPQLLQ
CAV2     NHFFSRAALVGKVELNDTGTAATGFT.....NWNIDIMGYAQL.RRKLEMFTYMRFNAEFTFVATTRA.......GRVPSRVLQ
CAV9     ENFLGRSACVYMEEYKTTDKHVN...KKP.VAWPINTKQMVQM.RRKLEMFTYLRFDMEVTFVITSRQDPGTTLAQDMPVLTRQ
CBV1     ENFLCRSACVYYATYNNNSEKG...YAE....WVINTRQVAQLLRRKLEF.TYLRFDLELTFVITSAQEPSTATSVDAPVQTQQ
CBV3     ENFLCRSACVYFTEYKNSGAKR...YAE....WVLTPRQAAQL.RRKLEFFTYVRFDLELTFVITSTQQPSTTQNQDAQILTHQ
CBV4     ENFLCRSACVIYIKYSSAESNNLKRYAE....WVINTRQVAQL.RRKMEMFTYIRCDMELTFVITSHQEMSTATNSDVPVQTHQ
CBV5     ENFLCRSACVYYTTYKNHGTDGNFAY......WVINTRQVAQL.RRKLEMFTYARFDLELTFVITSTQEQSTIQGQDSPVLTHQ
Echo6    ENFLSRSACVYIVEYKTQDTTPD....KMYDSWVINTRQVAQL.RRKLEFFTYVRFDVEVTFVITSVQDDSTRQNTDTPVLTHQ
Echo9    ENFLCRSACVRMAKYEARGNLKA....LTLDAWEISVRDMVQL.RRKCEMFTYLRFDVEVTFVITSYQRQGTSSIQICPYDAHQ
Echo11   ENFLSRSACVYMGGYHTTNTDQT....KLFASWTISARRMVQM.RRKLEIFTYVRFDVEVTFVITSKQDQGSRLGQDMPPLTHQ
Echo12   ENFLCRAACVCITKYKTKDSDPV....QRYANWRINTRQMAQL.RRKFELFTYLRFDMEVTFVITSSQDDGTQLAQDMPVLTHQ
EV70     ENFLGRSALVCMRSFEYKNHSTSTSSIQKNFFVWTLNTRELVQIRRKMELFTYLRFDTEITIVPTLRLFSSSNASSSGLPNLTLQ
EV71     DSFFSRAGLVGEIDLPL... EGTTNPNGYANWDIDITGYAQ.MRRKVELFTYMRFDAEFTFV.......ACTPTGEVVP.QLLQ
```

FIGURE 2 (1 of 2)

B)

```
CAV24    LMYIPPGAPRPTAWDDYTWQSSSNPSVFYTYGSAPPRISIPYVGIANAYSHFYDGFARVPLKDETVDSGDTYYGLVTI
CAV21    IMYIPPGAPRPSSWDDYTWQSSSNPSIFYMYGNAPPRMSIPYVGIANAYSHFYDGFARVPLEGENTDAGDTFYGLVSI
CAV16    YMYVPPGAPKPTSRDSFAWQTATNPSIFVKLTDPPAQVSVPFMSPASAYQWFYDGYPTFGAHPQSNDADYGQCP....
CAV2     YMYVPPGAPKPDGREAFQWQSSTNPSVFSXMTDPPPQVSVPFMSPASAYHGFYDGYPTFGEHNGEDSLRTGNA.....
CAV9     IMYVPPGGPIPAKVDDYAWQTSTNPSIFWTEGNAPARMSIPFISIGNAYSNFYDGWSNFDQRGSYGYNTL........
CBV1     IMYVPPGGPVPTKVTDYAWQTSTNPSVFWTEGNAPPRMSIPFISIGNAYSCFYDGWTQFSRNGVYGINTL........
CBV3     IMYVPPGGPVPDKVDSYVWQTSTNPSVFWTEGNAPPRMSIPFLSIGNAYSNFYDGWSEFSRNGVYGINTL........
CBV4     IMYVPPGGPVPTSVNDYVWQTSTNPSIFWTEGNAPPRMSIPFMSIGNAYTMFYDGWSNFSRDGIYGYNSL........
CBV5     IMYVPPGGPVPTKINSYSWQTSTNPSVFWTEGSAPPRISIPFISIGNAYSMFYDGWAKFDKQGTYGINTL........
Echo6    IMYVPPGGPIPHAVDDYNWQTSTNPSVFWTEGNAPPRMSIPFMSVGNAYSNFYDGWSHFSQTGVYGFNTL........
Echo9    IMYIPPGGPIPKKVDGYEWQTSTNPSIFWTEGNAPPRMSIPFISIGNAYSSFYDGWSHFDSKGAYGFNTL........
Echo11   IMYIPPGGPIPKSVTDYAWQTSTNPSIFWTEGNAPPRMSIPFISIGNAYSNFYDGWSHFSQNGVYGYNTL........
Echo12   VMYIPPGGPVPNSATDFAWQSSTNPSIFWTEGNAPARMSIPFISIGNAYSNFYDGWSHFTQDGVYGFNSL........
EV70     VMYVPTGAPKPSSQDSFEWQSACNPSVFFKINDPPARLTIPFMSINSAYANFYDGFAGFEKKAT..DL....YGINPA
EV71     YMFVPPGAPKPESRESLAWQTATNPSVFVKLTDPPAQVSVPFMSPASAYQWFYDGYPTFGEHKQEKDLE...YGACP.

CAV24    NDFGTLAVRVVNEFNPARIISKIRVYMKPKHVRCWCPRPPRAVPY.RGEGVDFKQDSITPLIAVEN.INTF.................
CAV21    NDFGVLAVRAVNRSNPHTIHTSVRVYMKPKHIRCWCPRPPRAVLY.RGEGVDMISSAIQPLTKVDS.ITTF.................
CAV16    NNMMGTFSIRTVGTEKSPHSITLRVYMRIKHVRAWIPRPLRNQPYLFKTNPNYKGNDIKCTSTSRDKITTL.................
CAV2     NNALGTFSVRFVSEEITNERIIIRIYMRLKHIRAWVPRPLRSEPYVLKNFPNYTAVTHVTANRPSITNTGRF................
CAV9     NNLGHIYVRHVSGSSPHPITSTIRVYFKPKHTRAWVPRPPRLCQYKKAFSVDFTPTPITDTRKD...INTVAQSRRRGDMSTLNTHGAF
CBV1     NNMGTLYMRHVNEAGQGPIKSTVRIYFKPKHVKAWVPRPPRLCQYEKQKNVNFNPTGVTTTRSN...ITTT.................
CBV3     NNMGTLYARHVNAGSTGPIKSTIRIYFKFKHVKAWIPRPPRLCQYEKAKNVNFQPSGVTTTRQS...ITTM.................
CBV4     NNMGTIYARHVNDSSPGGLTSTIRIYFKPKHVKAYVPRPPRLCQYKKAKSVNFDVEAVTAERAS...LITT.................
CBV5     NNMGTLYMRHVNDGSPGPIVSTVRIYFKPKHVKTWVPRPPRLCQYQKAGNVNFEPTGVTESRTE...ITAM.................
Echo6    NNMGKLYFRHVNDRTISPITSKVRIYFKPKHVKAWVPRPPRLCEYTHKDNVDYSPKGVTTSRTS...ITITNSKHMETHGAF......
Echo9    NKMGHIYCRHVNKETPTKVTSYIRIYFKPKHVRAWVPRPPRLCQYMNKANVNFEATAVTDTRDT...INTVPLSTHGVSRGAY.....
Echo11   NHMGQIYVRHVNGSSPLPMTSTVRMYFKPKHVKAWVPRPPRLCQYKNASTVNFTPTNVTDKRTS...INYIPE...............
Echo12   NNMGSIYIRHVNEQSPYAITSTVRVYFKPKHVRAWVPRPPRLCAYEKSSNVNFKPTDVTTSRTS...ITEVPS...............
EV70     NTMGNLCLRVVNSYQPVQYTLTVRVYMKPKHIKAWAPRAPRTMPYTNILNNNYVGRSAAPNAPTAIVSDRSTIKTMPNDIDLTTA...
EV71     NNMMGTFSVRTVGSSKSKYPLVVRIYMRMKHVRAWIPRPMRNQNYLFKANPNYTGNSIKPTGTSR.............NAITTL...
```

FIGURE 2 (2 of 2)

## PARTIAL SCREENING OF NPEVs WITH PCR PRIMERS

| PRIMERS | SIZE(BP) | A9 | A12 | A21 | B1 | B2 | B3 | B4 | B5 | B6 | EC4 | EC11 | EC30 | EV7 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5S/6A* | 101 | + | - | - | + | + | + | + | + | + | + | + | + | - |
| 6S/7A | 134 | + | - | - | + | + | + | + | + | + | + | + | - | - |
| 7S/8A | 143 | - | - | - | - | - | + | + | + | - | - | - | - | - |
| 7S/9A* | 107 | - | - | - | + | + | + | + | + | + | + | + | + | - |
| 14S/11A* | 130 | - | - | - | + | + | + | + | + | + | + | + | + | - |
| 24S/47A | 77 | + | - | - | + | + | + | - | + | + | - | - | + | - |
| 24S/25A | 98 | + | - | - | - | + | - | - | - | - | - | - | - | - |
| 24S/46A | 98 | + | - | - | + | + | + | - | - | + | - | - | - | - |
| 34S/28A | 86 | - | - | + | - | - | - | - | - | - | - | - | - | - |
| 34S/33A | 89 | - | - | - | + | - | - | + | + | + | - | - | - | - |
| 34S/35A | 104 | - | - | - | - | - | - | - | - | + | - | + | - | - |
| 34S/38A | 101 | - | - | - | - | - | - | - | - | - | - | + | - | - |
| 34S/73A | 98 | + | - | - | - | - | - | - | - | - | - | + | - | - |
| 36S/35A | 80 | - | - | - | - | - | - | - | - | - | - | + | - | - |
| 39S/40A | 71 | - | + | - | - | - | - | - | - | - | - | - | - | + |
| 39S/41A | 62 | - | - | - | - | - | - | - | - | - | - | - | - | + |
| 51S/52A* | 83 | - | - | - | + | + | + | + | + | + | + | + | + | - |
| 55S/54A | 140 | - | - | - | - | - | - | - | - | - | - | - | - | + |
| 59S/27A | 152 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 61S/68A* | 104 | - | - | - | - | - | - | - | - | - | - | - | - | + |
| 62S/27A | 131 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 63S/43A | 80 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 64S/69A | 180 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 64S/65A* | 166 | - | - | - | - | - | - | - | - | - | - | - | - | + |
| 67S/1A* | 155 | - | - | - | - | - | - | - | - | - | - | + | - | - |
| 67S/8A* | 147 | - | - | - | - | - | - | - | - | - | - | + | - | - |

* = Primers selected for screening complete NPEV collection.

FIGURE 3

## NPEV PCR PRIMER POOL

| EV | 5S/6A | 7S/9A | 14S/11A | 51S/52A | 61S/68A | 64S/65A | 67S/1A | 67S/8A |
|---|---|---|---|---|---|---|---|---|
| A3 | - | - | - | - | + | - | + | - |
| A4 | - | - | - | - | + | - | - | - |
| A5 | - | - | - | - | + | - | - | - |
| A6 | - | - | - | - | + | - | - | - |
| A8 | - | - | - | - | + | + | + | - |
| A9 | + | - | - | - | - | - | - | - |
| A10 | - | - | - | - | + | + | - | - |
| A12 | - | - | - | - | - | - | - | - |
| A14 | - | - | - | - | - | + | - | - |
| A16 | - | - | - | - | + | + | - | - |
| A21 | - | - | - | - | - | - | - | - |
| A24 | - | - | - | - | - | - | - | - |
| B1 | + | + | + | + | - | - | - | - |
| B2 | + | + | + | + | - | - | - | - |
| B3 | + | + | + | + | - | - | - | - |
| B4 | + | + | + | + | - | - | - | - |
| B5 | + | + | + | + | - | - | - | - |
| B6 | + | + | + | + | - | - | - | - |
| EC3 | + | + | - | + | - | - | - | - |
| EC4 | + | + | + | + | - | - | - | - |
| EC5 | - | - | - | + | - | - | - | - |
| EC6 | - | + | + | + | - | - | - | - |
| EC7 | - | + | + | + | - | - | - | - |
| EC8 | - | - | - | + | - | - | - | - |
| EC9 | - | - | + | + | - | - | + | - |
| EC11 | + | + | + | + | - | - | + | + |
| EC12 | + | - | + | + | - | - | + | + |
| EC13 | + | + | - | + | - | - | + | - |
| EC14 | + | - | + | - | - | - | - | - |
| EC15 | + | - | - | + | - | - | - | - |
| EC16 | + | - | - | + | - | - | - | - |
| EC17 | + | + | - | + | - | - | + | + |
| EC18 | + | - | - | - | - | - | - | - |
| EC19 | + | + | + | + | - | - | - | + |
| EC20 | + | - | + | + | - | - | - | - |
| EC21 | + | - | + | + | - | - | + | + |
| EC24 | + | + | + | + | - | - | + | - |
| EC25 | + | - | + | + | - | - | - | - |
| EC26 | + | - | - | + | - | - | - | - |
| EC27 | + | + | - | + | - | - | - | - |
| EC29 | + | - | + | + | - | - | + | + |
| EC30 | + | + | + | + | - | - | - | - |
| EC31 | + | - | - | + | - | - | + | + |
| EC32 | + | - | + | + | - | - | - | - |
| EC33 | + | - | + | + | - | - | - | - |
| EV68 | - | - | - | - | - | - | - | - |
| EV69 | + | - | - | + | - | - | + | - |
| EV70 | - | - | - | - | + | - | - | - |
| EV71 | - | - | - | - | + | + | - | - |

FIGURE 4

## QUICK SCREEN CHART FOR NPEV PRIMER POOL*

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | |
|---|---|---|---|---|---|---|---|---|
| - | - | - | - | - | - | - | - | =A12, A21, A24, EV68 |
| - | - | - | - | - | + | - | - | =A14 |
| - | - | - | - | + | - | + | - | =A3 |
| - | - | - | - | + | - | - | - | =A4, A5, A6, EV70 |
| - | - | - | - | + | + | + | - | =A8 |
| + | - | - | - | - | - | - | - | =A9, EC18 |
| - | - | - | - | + | + | - | - | =A10, A16, EV71 |
| + | + | + | + | - | - | - | - | =B1, B2, B3, B4, B5, B6, EC4, EC30 |
| + | + | + | + | - | - | + | + | =EC11 |
| + | + | + | + | - | - | - | + | =EC19 |
| + | + | + | + | - | - | + | - | =EC24 |
| + | + | - | + | - | - | - | - | =EC3, EC27 |
| + | + | - | + | - | - | + | + | =EC17 |
| + | + | - | + | - | - | + | - | =EC13 |
| + | - | + | - | - | - | - | - | =EC14 |
| + | - | + | + | - | - | - | - | =EC20, EC25, EC32, EC33 |
| + | - | + | + | - | - | + | + | =EC12, EC21, EC29 |
| + | - | - | + | - | - | + | + | =EC31 |
| + | - | - | + | - | - | - | - | =EC15, EC16, EC26 |
| + | - | - | + | - | - | + | - | =EV69 |
| - | + | + | + | - | - | - | - | =EC6, EC7 |
| - | - | + | + | - | - | + | - | =EC9 |
| - | - | - | + | - | - | - | - | =EC5, EC8 |

*Lane 1=5S/6A; 2=7S/9A; 3=14S/11A; 4=51S/52A; 5=61S/68A; 6=64S/65A 7=67S/1A; 8=67S/8A.

FIGURE 5

# INTERNATIONAL SEARCH REPORT

Inte ional Application No
PCT/US 97/17734

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 6 C12Q1/70 C12Q1/68 //C07K14/085

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category * | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
| --- | --- | --- |
| A | WO 95 02704 A (US GOVERNMENT) 26 January 1995<br>see page 3, paragraph 1<br>see page 22, line 5 - page 25, line 15<br>--- | 1-3, 8-10, 12-16 |
| A | WO 90 11376 A (UNIVERSITY PATENTS INC) 4 October 1990<br>see the whole document<br>--- | 1-3, 8-10, 12-16 |
| A | FR 2 623 817 A (PASTEUR INSTITUT ;CENTRE NAT RECH SCIENT (FR); INST NAT SANTE RECH) 2 June 1989<br>see the whole document<br>---<br>-/-- | 1-3 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

| Date of the actual completion of the international search | Date of mailing of the international search report |
| --- | --- |
| 6 March 1998 | 25/03/1998 |

| Name and mailing address of the ISA | Authorized officer |
| --- | --- |
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Osborne, H |

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | EGGER D ET AL: "Reverse transcription multiplex PCR for differentiation between polio- and enteroviruses from clinical and environmental samples" JOURNAL OF CLINICAL MICROBIOLOGY, vol. 33, no. 6, June 1995, pages 1442-7, XP002058058 see the whole document | 1-3, 8-10, 12-16 |
| A | MUSCILLO M ET AL: "Detection of enteroviruses in cellular lysates by RT-PCR: Differentiation between poliovirus and nonpolioviruses" L'IGIENE MODERNA, vol. 103 , no. 2 , 1995, pages 223-36, XP002058059 see summary | 1-3, 8-10, 12-16 |
| A | EP 0 434 992 A (MAX PLANCK GESELLSCHAFT) 3 July 1991 | |

# INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/US 97/17734

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9502704 A | 26-01-95 | US 5585477 A | 17-12-96 |
| | | AU 7332494 A | 13-02-95 |
| | | EP 0789782 A | 20-08-97 |
| | | JP 9500530 T | 21-01-97 |
| | | US 5691134 A | 25-11-97 |
| WO 9011376 A | 04-10-90 | US 5075212 A | 24-12-91 |
| | | AT 161891 T | 15-01-98 |
| | | AU 5564090 A | 22-10-90 |
| | | DE 69031911 D | 12-02-98 |
| | | EP 0465603 A | 15-01-92 |
| FR 2623817 A | 02-06-89 | NONE | |
| EP 0434992 A | 03-07-91 | DE 3939200 A | 29-05-91 |
| | | JP 3183483 A | 09-08-91 |

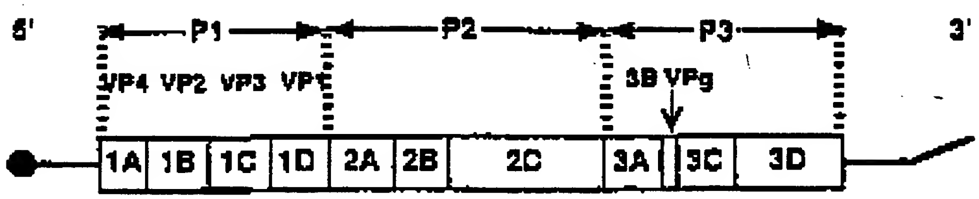


FIGURE 1

# NPEV VP1 AMINO ACID ALIGNMENT

A)

```
CAV24    GIEETIDTVISNALQLSQPKPQKQLTAQSTPSTS....GVNSQEVPALTAVETGVSGQAIPSDVIETRHVVNYKTRSESTL
CAV21    GIEDLIDTAIKNALRVSQPLAPSQLKQ.........PNGVNSQEVPALTAVETGASGQAIPSDVVETRHVINYKTRSESCL
CAV16    GDGIADMIDQAVTSRVGRALTSLQVEPTAANTHASE.HRLGTGLVPALQAAETGASSNAQDENLIETRCVLNHHSTQETTI
CAV2     GDGIEDAITHTVNATINRVLDRPISHSSTAANTQVSQHSIETGRVPALQAAETGATSNASDENLIETRCVVNHHSVEEASL
CAV9     GDVEEAIERARCTVADTMRTGPS.................NSASVPALTAVETGHTSQVTPSDTMQTRHVKNYHSRSESTV
CBV1     GPVEESVERAMVRVADTVSSKPT.................NSESIPALTAAETGHTSQVVPSDTMQTRHVKNYHSRSESSI
CBV3     GPVEDAITAAIGRVADTVGTGPT.................NSEAIPALTAAETGHTSQVVPGDTMQTRHVKNYHSRSESTI
CBV4     GPTEESVERAMGRVADTIARGPS.................NSEQIPALTAVETGHTSQVDPSDTMQTRHVHNYHSRSESSI
CBV5     GPPGEAVERAIARVADTISSGPV.................NSESIPALTAAETGHTSQVVPADTMQTRHVKNYHSRSESTV
Echo6    NDVQNAVERSIVRVADTLPSGPS.................NSESIPALTAAETGHTSQVVPSDTIQTRHVRNFHVRSESSV
Echo9    SDVREAVEGAIGRVADTIRSGPS.................NSEAVPALTAAETGHTSQVVPSDTMQTRHVKNYHSRSESTI
Echo11   GDVVEAVENAVARVADTIGSGPS.................NSQAVPALTAVETGHTSQVTPSDTMQTRHVKNYHSRSESSI
Echo12   GDVEEAVNRAVARVADTLPTGPR.................NSESIPALTAAETGHTSQVVPGDTMQTRHVKNYHSRTESSV
EV70     GEIVKTVANTVE........................SEIKAELGVIPSLNAVETGATSNTEPEEAIQTRTVINMHGTAECLV
EV71     GDRVADVIESSIGDSVSRALTQALPAPTGQNTQVS.SHRLDTGEVPALQAAEIGASSNTSDESMIETRCVLNSHSTAETTL

CAV24    ESFFGRSACVTMLEVENFHATTEADKKKQFTTWAITYTDTVQL.RRKLEFFTYSRFDLEMTFVITERYYTSNT..GYARNQVYQ
CAV21    ESFFGRAACVTILSLTNSSKSGEE..KKHFNIWNITYTDTVQL.RRKLEFFTYSRFDLEMTFVFTENYPSTAS..GEVRNQCDQ
CAV16    GNFFSRAGLVSIITMPTTGTQNTDGY....VNWDIDLMGYAQM.RRKCELFTYMRFDAEFTFVAAKPN.......GELVPQLLQ
CAV2     NNFFSRAALVGKVELNDTGTAATGFT.....NWNIDIMGYAQL.RRKLEMFTYMRFNAEFTFVATTRA.......GRVPSRVLQ
CAV9     ENFLGRSACVYMEEYKTTDKEVN...KKF.VAWPINTKQMVQM.RRKLEMFTYLRFDMEVTFVITSRQDPGTTLAQDMPVLTRQ
CBV1     ENFLCRSACVYYATYNNHSEKG...YAE....WVINTRQVAQLLRRKLEF.TYLRFDLELTFVITSAQEPSTATSVDAPVQTQQ
CBV3     ENFLCRSACVYFTEYKNSGAKR...YAE....WVLTPRQAAQL.RRKLEFFTYVRFDLELTFVITSTQQPSTTQNQDAQILTHQ
CBV4     ENFLCRSACVIYIKYSSAESHNLKRYAE....WVINTRQVAQL.RRKMEMFTYIRCDMELTFVITSHQEMSTATNSDVPVQTHQ
CBV5     ENFLCRSACVYYTTYKNHGTDGNFAY......WVINTRQVAQL.RRKLEMFTYARFDLELTFVITSTQEQSTIQGQDSPVLTHQ
Echo6    ENFLSRSACVYIVEYKTQDTTPD....SMYDSWVINTRQVAQL.RRKLEFFTYVRFDVEVTFVITSVQDDSTRQNTDTPVLTHQ
Echo9    ENFLCRSACVRMAKYEARGNLKA....LTLQAWEISVRDMVQL.RRKCEMFTYLRFDVEVTFVITSYQRQGTSSIQIC?YDAHQ
Echo11   ENFLSRSACVYHGGYETTNTDQT....KLFASWTISARRMVQM.RRKLEIFTYVRFDVEVTFVITSKQDQGSRLGQDMPPLTHQ
Echo12   ENFLCRAACVCITKYKTKDGDPV....QRYANWAINTRQMAQL.RRKFELFTYLRFDMEVTFVITSSQDDGTQLAQDMPVLTHQ
EV70     ENFLCRSALVCMRSFEYKNNSTSTSSIQKNYFVWTLNTRELVQIRRKMELFTYLRFDTEITIVPTLRLFSSSNASSSGLPNLTLQ
EV71     DSFFSRAGLVGEIDLPL... EGTTNPNGYANWDIDITGYAQ.MRRKVELFTYMRFDAEFTFV.......ACTPTGEVVP.QLLQ
```

FIGURE 2 (1 of 2)

B)

```
CAV24     LNYIPPGAPRPTAMDDYTWQSSSNPSVFFTYGSAPPRISIPYVGIANAYSHFYDGFARVPLKDETVDSGDTYYGLVTI
CAV21     INYIPPGAPRPSSWDDYTWQSSSNPSIFYMYGNAPPRMSIPYVGIANAYSHFYDGFARVPLEGENTDAGDTFYGLVSI
CAV16     THYVPPGAPKPTSRDSFAWQTATNPSIFVKLTDPPAQVSVPFMSPASAYQWFYDGYPTFGAHPQSNDADYGQCP....
CAV2      THYVPPGAPKPDGREAFQWQSSTNPSVFSSMTDPPPQVSVPFMSPASAYHGFYDGYPTFGEHNGEDSLRTGNA.....
CAV9      IMYVPPGGPIPAKVDDYAWQTSTNPSIFWTEGNAPARMSIPFISIGNAYSNFYDGWSNFDQRGSYGYNTL........
CBV1      IMYVPPGGPVPTKVTDYAWQTSTNPSVFWTEGNAPPRMSIPFISIGNAYSCPYDGWTQFSRNGVYGINTL........
CBV3      IHYVPPGGPVPDKVDSYVWQTSTNPSVFWTEGNAPPRMSIPFLSIGNAYSNFYDGWSEFSRNGVYGINTL........
CBV4      IMYVPPGGPVPTSVNDYVWQTSTNPSIFWTEGNAPPRMSIPFMSIGNAYTNFYDGWSNFSRDGIYGYNSL........
CBV5      IHYVPPGGPVPTKINSYSWQTSTNPSVFWTEGSAPPRISIPFISIGNAYSNFYDGWAKFDKQGTYGINTL........
Echo6     IMYVPPGGPIPHAVDDYNWQTSTNPSVFWTEGNAPPRMSIPFMSVGNAYSNFYDGWSNFSQTGVYGFNTL........
Echo9     IMYIPPGGPIPKKVDGYEWQTSTNPSIFWTEGNAPPRMSIPFISIGNAYSSFYDGWSNFDSKGAYGFNTL........
Echo11    IMYIPPGGPIPKSVTDYAWQTSTNPSIFWTEGNAPPRMSIPFISIGNAYSNFYDGWSNFSQNGVYGYNTL........
Echo12    VHYIPPGGPVPNSATDPAWQSSTNPSIFWTEGNAPARMSIPFISIGNAYSNFYDGWSNFTQDGVYGFNSL........
EV70      VMYVPTGAPKPSSQDSFEWQSACNPSVFFKINDPPARLTIPFMSTNSAYANFYDGFAGFEKKAT..DL....YGINPA
EV71      YHFVPPGAPKPESRESLAWQTATNPSVFVKLTDPPAQVSVPFMSPASAYQWFYDGYPTFGEHKQEKDLE...YGACP.

CAV24     KDFGTLAVRVVNEFNPARIISKIRVYMKPKHVRCWCPRPPRAVPY.RGEGVDFKQDSITPLIAVEN.INTF.................
CAV21     KDFGVLAVRAVNRSNPETIHTSVRVYMKPKHIRCWCPRPPRAVLY*.RGEGVDMISSAIQPLTKVDS.ITTF................
CAV16     NNMGTPSIRTVGTEKSPHSITLAVYMRIKHVRAWIPRPLRNQPYLFKTNPNYKGNDIKCTSTSRDKITTL.................
CAV2      NNALGTPSVRPVSEEITNERIIIRIYMRLKHIRAWVPRPLRSEPYVLKNFPNYTAVTHVTANRPSITNTGRF................
CAV9      NMLGHIYVRHVSGSSPHPITSTIRVYFKPKHTRAWVPRPPRLCQYKKAFSVDFTPTPITDTRKD...INTVAQSRRRGDMSTLNTHGAF
CBV1      NNMGTLYMRHVNEAGQGPIHSTVRIYFKPKHVKAWVPRPPRLCQYEKQKNVNFNPTGVTTTRSN...TTTT.................
CBV3      NNMGTLYARHVNAGSTGPIHSTIRIYFKPKHVKAWIPRPPRLCQYEKAKNVNFQPSGVTTTRQS...ITTM.................
CBV4      NNMGTIYARHVNDSSPGGLTSTIRIYFKPKHVKAYVPRPPRLCQYKKAKSVNFDVEAVTAERAS...LITT.................
CBV5      NNMGTLYMRHVNDGSPGPIVSTVRIYFKPKHVKTWVPRPPRLCQYQKAGNVNFEPTGVTESRTE...ITAM.................
Echo6     NNMGKLYFRHVNDRTISPITSKVRIYFKPKHVKAWVPRPPRLCEYTHKDNVDYEPKGVTTSRTS...ITITNSKHNETHGAF.......
Echo9     NRMGHTYCRHVNKETPTKVTSYIRIYFKPKHVRAWVPRPPRLCQYMNKANVNFEATAVTDTRDT...INTVPLSTHGVSRGAY......
Echo11    NNMGQIYVRHVNGSSPLPMTSTVRMYFKPKHVKAWVPRPPRLCQYKNASTVNFTPTNVTDKRTS...INYIPE................
Echo12    NNMGSIYIRHVNEQSPYAITSTVRVYFKPKHVRAWVPRPPRLCAYEKSSNVNFKPTDVTTSRTS...ITEVPS................
EV70      NTMGHLCLAVVNSYQPVQYTLTVRVYMKPKHIKAWAPRAPRTMPYTNILNNNYVGRSAAPNAPTAIVSDRSTIKTMPNDIDLTTA...
EV71      NNMGTFSVRTVGSSKSKYPLVVRIYMRMKHVRAWIPRPMRNQNYLFKANPNYTGNSIKPTGTSR..............NAITTL...
```

FIGURE 2 (2  f 2)

PARTIAL SCREENING OF NPEVs WITH PCR PRIMERS

| PRIMERS | SIZE(BP) | A9 | A12 | A21 | B1 | B2 | B3 | B4 | B5 | B6 | EC6 | EC11 | EC30 | EV7 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 5S/6A* | 101 | + | - | - | + | + | + | + | + | + | + | + | + | - |
| 6S/7A | 134 | + | - | - | + | + | + | + | + | + | + | + | - | - |
| 7S/8A | 143 | - | - | - | - | - | + | + | + | - | - | - | - | - |
| 7S/9A* | 107 | - | - | - | + | + | + | + | + | + | + | + | + | - |
| 14S/11A* | 130 | - | - | - | + | + | + | + | + | + | + | + | + | - |
| 24S/47A | 77 | + | - | - | + | + | + | - | + | + | - | - | + | - |
| 24S/25A | 98 | + | - | - | - | + | - | - | - | - | - | - | - | - |
| 24S/46A | 98 | + | - | - | + | + | + | - | - | + | - | - | - | - |
| 34S/28A | 86 | - | - | + | - | - | - | - | - | - | - | - | - | - |
| 34S/33A | 89 | - | - | - | + | - | - | + | + | + | - | - | - | - |
| 34S/35A | 104 | - | - | - | - | - | - | - | - | + | - | + | - | - |
| 34S/38A | 101 | - | - | - | - | - | - | - | - | - | - | + | - | - |
| 34S/73A | 98 | + | - | - | - | - | - | - | - | - | - | + | - | - |
| 36S/35A | 80 | - | - | - | - | - | - | - | - | - | - | + | - | - |
| 39S/40A | 71 | - | + | - | - | - | - | - | - | - | - | - | - | + |
| 39S/41A | 62 | - | - | - | - | - | - | - | - | - | - | - | - | + |
| 51S/52A* | 83 | - | - | - | + | + | + | + | + | + | + | + | + | - |
| 55S/54A | 140 | - | - | - | - | - | - | - | - | - | - | - | - | + |
| 59S/27A | 152 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 61S/68A* | 104 | - | - | - | - | - | - | - | - | - | - | - | - | + |
| 62S/27A | 131 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 63S/43A | 80 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 64S/69A | 180 | - | - | - | - | - | - | - | - | - | - | - | - | - |
| 64S/65A* | 166 | - | - | - | - | - | - | - | - | - | - | - | - | + |
| 67S/1A* | 155 | - | - | - | - | - | - | - | - | - | - | + | - | - |
| 67S/8A* | 147 | - | - | - | - | - | - | - | - | - | - | + | - | - |

* = Primers selected for screening complete NPEV collection.

FIGURE 3

NPEV PCR PRIMER POOL

| EV | 5S/6A | 7S/9A | 14S/11A | 51S/52A | 61S/68A | 64S/65A | 67S/1A | 67S/8A |
|---|---|---|---|---|---|---|---|---|
| A3 | - | - | - | - | + | - | + | - |
| A4 | - | - | - | - | + | - | - | - |
| A5 | - | - | - | - | + | - | - | - |
| A6 | - | - | - | - | + | - | - | - |
| A8 | - | - | - | - | + | + | + | - |
| A9 | + | - | - | - | - | - | - | - |
| A10 | - | - | - | - | + | + | - | - |
| A12 | - | - | - | - | - | - | - | - |
| A14 | - | - | - | - | - | + | - | - |
| A16 | - | - | - | - | + | + | - | - |
| A21 | - | - | - | - | - | - | - | - |
| A24 | - | - | - | - | - | - | - | - |
| B1 | + | + | + | + | - | - | - | - |
| B2 | + | + | + | + | - | - | - | - |
| B3 | + | + | + | + | - | - | - | - |
| B4 | + | + | + | + | - | - | - | - |
| B5 | + | + | + | + | - | - | - | - |
| B6 | + | + | + | + | - | - | - | - |
| EC3 | + | + | - | + | - | - | - | - |
| EC4 | + | + | + | + | - | - | - | - |
| EC5 | - | - | - | + | - | - | - | - |
| EC6 | - | + | + | + | - | - | - | - |
| EC7 | - | + | + | + | - | - | - | - |
| EC8 | - | - | - | + | - | - | - | - |
| EC9 | - | - | + | + | - | - | + | - |
| EC11 | + | + | + | + | - | - | + | + |
| EC12 | + | - | + | + | - | - | + | + |
| EC13 | + | + | - | + | - | - | + | - |
| EC14 | + | - | + | - | - | - | - | - |
| EC15 | + | - | - | + | - | - | - | - |
| EC16 | + | - | - | + | - | - | - | - |
| EC17 | + | + | - | + | - | - | + | + |
| EC18 | + | - | - | - | - | - | - | - |
| EC19 | + | + | + | + | - | - | - | + |
| EC20 | + | - | + | + | - | - | - | - |
| EC21 | + | - | + | + | - | - | + | + |
| EC24 | + | + | + | + | - | - | + | - |
| EC25 | + | - | + | + | - | - | - | - |
| EC26 | + | - | - | + | - | - | - | - |
| EC27 | + | + | - | + | - | - | - | - |
| EC29 | + | - | + | + | - | - | + | + |
| EC30 | + | + | + | + | - | - | - | - |
| EC31 | + | - | - | + | - | - | + | + |
| EC32 | + | - | + | + | - | - | - | - |
| EC33 | + | - | + | + | - | - | - | - |
| EV68 | - | - | - | - | - | - | - | - |
| EV69 | + | - | - | + | - | - | + | - |
| EV70 | - | - | - | - | + | - | - | - |
| EV71 | - | - | - | - | + | + | - | - |

FIGURE 4

QUICK SCREEN CHART FOR NPEV PRIMER POOL*

| 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | |
|---|---|---|---|---|---|---|---|---|
| - | - | - | - | - | - | - | - | =A12, A21, A24, EV68 |
| - | - | - | - | - | + | - | - | =A14 |
| - | - | - | - | + | - | + | - | =A3 |
| - | - | - | - | + | - | - | - | =A4, A5, A6, EV70 |
| - | - | - | - | + | + | + | - | =A8 |
| + | - | - | - | - | - | - | - | =A9, EC18 |
| - | - | - | - | + | + | - | - | =A10, A16, EV71 |
| + | + | + | + | - | - | - | - | =B1, B2, B3, B4, B5, B6, EC4, EC30 |
| + | + | + | + | - | - | + | + | =EC11 |
| + | + | + | + | - | - | - | + | =EC19 |
| + | + | + | + | - | - | + | - | =EC24 |
| + | + | - | + | - | - | - | - | =EC3, EC27 |
| + | + | - | + | - | - | + | + | =EC17 |
| + | + | - | + | - | - | + | - | =EC13 |
| + | - | + | - | - | - | - | - | =EC14 |
| + | - | + | + | - | - | - | - | =EC20, EC25, EC32, EC33 |
| + | - | + | + | - | - | + | + | =EC12, EC21, EC29 |
| + | - | - | + | - | - | + | + | =EC31 |
| + | - | - | + | - | - | - | - | =EC15, EC16, EC26 |
| + | - | - | + | - | - | + | - | =EV69 |
| - | + | + | + | - | - | - | - | =EC6, EC7 |
| - | - | + | + | - | - | + | - | =EC9 |
| - | - | - | + | - | - | - | - | =EC5, EC8 |

*Lane 1=5S/6A; 2=7S/9A; 3=14S/11A; 4=51S/52A; 5=61S/68A; 6=64S/65A 7=67S/1A; 8=67S/8A.

FIGURE 5

PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12Q 1/70, 1/68 // C07K 14/085

A3

(11) International Publication Number: WO 98/14611

(43) International Publication Date: 9 April 1998 (09.04.98)

(21) International Application Number: PCT/US97/17734

(22) International Filing Date: 1 October 1997 (01.10.97)

(30) Priority Data:
60/027,353 2 October 1996 (02.10.96) US

(71) Applicant (*for all designated States except US*): THE GOVERNMENT OF THE UNITED STATES OF AMERICA, represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; The Centers for Disease Control and Prevention, 1600 Clifton Road N.E., Atlanta, GA 30333 (US).

(72) Inventor; and
(75) Inventor/Applicant (*for US only*): KILPATRICK, David [US/US]; 1095 Fulton Court, Norcross, GA 30093-4041 (US).

(74) Agents: SAMPLES, Kenneth, H. et al.; Fitch, Even, Tabin & Flannery, Suite 900, 135 South LaSalle Street, Chicago, IL 60603-4277 (US).

(81) Designated States: AU, CA, JP, US, European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE).

**Published**
*With international search report.*

(88) Date of publication of the international search report: 28 May 1998 (28.05.98)

(54) Title: DETECTION AND IDENTIFICATION OF NON-POLIO ENTEROVIRUSES

(57) Abstract

This invention provides sensitive nucleic acid hybridization assay methods and kits for the detection of non-polio enterovirus nucleic acids. The methods are particularly useful in detecting the presence of enterovirus nucleic acids in a biological sample, and for ascertaining the serotype of enteroviruses present in a sample.

*FOR THE PURPOSES OF INFORMATION ONLY*

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| AL | Albania | ES | Spain | LS | Lesotho | SI | Slovenia |
| AM | Armenia | FI | Finland | LT | Lithuania | SK | Slovakia |
| AT | Austria | FR | France | LU | Luxembourg | SN | Senegal |
| AU | Australia | GA | Gabon | LV | Latvia | SZ | Swaziland |
| AZ | Azerbaijan | GB | United Kingdom | MC | Monaco | TD | Chad |
| BA | Bosnia and Herzegovina | GE | Georgia | MD | Republic of Moldova | TG | Togo |
| BB | Barbados | GH | Ghana | MG | Madagascar | TJ | Tajikistan |
| BE | Belgium | GN | Guinea | MK | The former Yugoslav Republic of Macedonia | TM | Turkmenistan |
| BF | Burkina Faso | GR | Greece | | | TR | Turkey |
| BG | Bulgaria | HU | Hungary | ML | Mali | TT | Trinidad and Tobago |
| BJ | Benin | IE | Ireland | MN | Mongolia | UA | Ukraine |
| BR | Brazil | IL | Israel | MR | Mauritania | UG | Uganda |
| BY | Belarus | IS | Iceland | MW | Malawi | US | United States of America |
| CA | Canada | IT | Italy | MX | Mexico | UZ | Uzbekistan |
| CF | Central African Republic | JP | Japan | NE | Niger | VN | Viet Nam |
| CG | Congo | KE | Kenya | NL | Netherlands | YU | Yugoslavia |
| CH | Switzerland | KG | Kyrgyzstan | NO | Norway | ZW | Zimbabwe |
| CI | Côte d'Ivoire | KP | Democratic People's Republic of Korea | NZ | New Zealand | | |
| CM | Cameroon | | | PL | Poland | | |
| CN | China | KR | Republic of Korea | PT | Portugal | | |
| CU | Cuba | KZ | Kazakstan | RO | Romania | | |
| CZ | Czech Republic | LC | Saint Lucia | RU | Russian Federation | | |
| DE | Germany | LI | Liechtenstein | SD | Sudan | | |
| DK | Denmark | LK | Sri Lanka | SE | Sweden | | |
| EE | Estonia | LR | Liberia | SG | Singapore | | |

## INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/17734

**A. CLASSIFICATION OF SUBJECT MATTER**
IPC 6 C12Q1/70 C12Q1/68 //C07K14/085

According to International Patent Classification (IPC) or to both national classification and IPC

**B. FIELDS SEARCHED**

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

| Category ° | Citation of document, with indication, where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | WO 95 02704 A (US GOVERNMENT) 26 January 1995<br>see page 3, paragraph 1<br>see page 22, line 5 - page 25, line 15<br>--- | 1-3, 8-10, 12-16 |
| A | WO 90 11376 A (UNIVERSITY PATENTS INC) 4 October 1990<br>see the whole document<br>--- | 1-3, 8-10, 12-16 |
| A | FR 2 623 817 A (PASTEUR INSTITUT ;CENTRE NAT RECH SCIENT (FR); INST NAT SANTE RECH) 2 June 1989<br>see the whole document<br>---<br>-/-- | 1-3 |

[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

2

| Date of the actual completion of the international search | Date of mailing of the international search report |
|---|---|
| 6 March 1998 | 25/03/1998 |

| Name and mailing address of the ISA | Authorized officer |
|---|---|
| European Patent Office, P.B. 5818 Patentlaan 2<br>NL - 2280 HV Rijswijk<br>Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,<br>Fax: (+31-70) 340-3016 | Osborne, H |

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

| Category ° | Citation of document, with indication,where appropriate, of the relevant passages | Relevant to claim No. |
|---|---|---|
| A | EGGER D ET AL: "Reverse transcription multiplex PCR for differentiation between polio- and enteroviruses from clinical and environmental samples" JOURNAL OF CLINICAL MICROBIOLOGY, vol. 33, no. 6, June 1995, pages 1442-7, XP002058058 see the whole document --- | 1-3, 8-10, 12-16 |
| A | MUSCILLO M ET AL: "Detection of enteroviruses in cellular lysates by RT-PCR: Differentiation between poliovirus and nonpolioviruses" L'IGIENE MODERNA, vol. 103 , no. 2 , 1995, pages 223-36, XP002058059 see summary --- | 1-3, 8-10, 12-16 |
| A | EP 0 434 992 A (MAX PLANCK GESELLSCHAFT) 3 July 1991 ----- | |

2

| Patent document cited in search report | Publication date | Patent family member(s) | Publication date |
|---|---|---|---|
| WO 9502704 A | 26-01-95 | US 5585477 A | 17-12-96 |
| | | AU 7332494 A | 13-02-95 |
| | | EP 0789782 A | 20-08-97 |
| | | JP 9500530 T | 21-01-97 |
| | | US 5691134 A | 25-11-97 |
| WO 9011376 A | 04-10-90 | US 5075212 A | 24-12-91 |
| | | AT 161891 T | 15-01-98 |
| | | AU 5564090 A | 22-10-90 |
| | | DE 69031911 D | 12-02-98 |
| | | EP 0465603 A | 15-01-92 |
| FR 2623817 A | 02-06-89 | NONE | |
| EP 0434992 A | 03-07-91 | DE 3939200 A | 29-05-91 |
| | | JP 3183483 A | 09-08-91 |